AD_____

Award Number: DAMD17-00-1-0249

TITLE: FCCC Institutional Breast Cancer Training Program (FCCC-IBCTP)

PRINCIPAL INVESTIGATOR: Jose Russo, M.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual Summary (1 Jul 2002 - 30 Jun 2003)

4. TITLE AND SUBTITLE
FCCC Institutional Breast Cancer Training Program (FCCC-IBCTP)

5. FUNDING NUMBERS
DAMD17-00-1-0249

6. AUTHOR(S)
Jose Russo, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

E-Mail: J_Russo@fccc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The present report covers the fork performed by four different trainees working in: ***i-Predispostion to genomic instability in breast cancer: analysis of molecular mechanisms.*** The goal of this research is to better understand intrinsic genetic factor leading to genome instability, with the intent of improving diagnosis and treatment for breast cancer. ***ii-Proline 47 is important in mediating p53-dependent apoptosis.*** We are studying a rare polymorphism of p53 that changes the praline. Residue at amino acid 47 to Serine. ***iii- Development of a Mouse Model for the Targeted Disruption of the Appl Gene in Mammary Gland.*** To date, APPL is the only adaptor reported to interact with AKT kinases. Since AKT2 appears to play a more prominent role in human neoplasia than other members of he AKT family, the characterization of APPL may provide important insights regarding oncogenic mechanisms involving AKT2. ***iv-Cloning of a new gene/s in chromosome 17p13.2-13.1 that control apoptosis.*** The aims are to isolate in the precise location in chromosome 17p13.2-p13.1 the gene(s) responsible for the control of Fas-receptor ligand complex function and to test the cloned gene/s functionality in cell transformation by cDNA transfection studies in vitro.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Chromosome 17, fax, p53, AKT2, Proline 47 and Appl.	35
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

A-INTRODUCTION

The focus of the FCCC Institutional Breast Cancer Training Program (IBCTP) is to integrate the unique talents and interests of the Center's basic scientists, clinical investigators and behavioral scientists to create a comprehensive effort to approach the problems of breast cancer. The rich scientific and intellectual environment of FCCC is nurtured by a cohesive interdisciplinary program that is based on expertise in areas of high relevance to breast cancer. The Institutional Breast Cancer Training Program offers to the postdoctoral trainees practical experience in the fields of cellular and molecular biology, drug resistance and targeted immunotherapy, genetic epidemiology and control, psychosocial and behavioral medicine, as well as breast cancer prevention, diagnosis, and treatment.

B-BODY

B-i. Organization of the FCCC-Institutional Breast Cancer Training Program. Following our statement of work we have accomplished the following tasks:

Task 3. During the third year of the program we have been implementing the training phase of the program by monitoring each of the individual projects and selected a second roster of four new trainees. The trainees have been attending at least one general lecture a week from those offered by the Fox Chase Cancer Center and a special seminar targeted to the formation of the trainees addressing critical subjects in breast cancer. We have established a half-day seminar twice a year in which the trainees present their work in front of the Faculty and the Advisory Panel.

Task 4. Applicant selection for the second cycle started on July 1, 2002. Using the web page http://www.fccc.edu/postdoc/BreastCaTraining.html developed by our *Recruitment Panel* formed by our Faculty members we have received 32 aplications for this second cycle that started on January 1st, 2003. Another source is the contacted scientists in the breast cancer area by phone and e-mail. All the applicants were required to present a statement of the fellow's background, training, and professional interests and goals, and a minimum of three recommendation letters. Fellows applying to the program must have a Ph.D. or M.D. degree with background in biology, molecular biology; chemistry, including organic and physical chemistry; mathematics; biochemistry; genetics, and or behavioral sciences.

Important evaluation criteria used by *The Recruitment Panel* and the *Applications Evaluation Committee* is the evaluation of personal statements of research and career goals, previous laboratory research experience, publications, and the recommendation letters submitted. From the 32 applicants we have selected only four.

The photograph shows in the first road from left to right: H. You, S. V .Fernandez, J. Russo, E. Pugacheva and K. Sherman. In the second road shows from left to right: X. Li; F. Zalatoris, S. Pan and H.M.Lareef.

The first four that have completed their training in December 31st , 2002 are Dr. F. Zalatoris, X. Pan, H.M. Lareef and K. Sherman; and the new four ones that have started this January 1st, 2003, are Dr. Elena Pugacheva, Dr Xiaoxian Li, Dr. Huihong You and Dr. Sandra V. Fernandez.

Trainee: Elena Pugacheva, PhD
Mentor: Erica Golemis, Ph.D.
Period reported: January 1, 2003 – to July 30, 2003

Introduction:

The goal of this research is to better understand intrinsic genetic factors leading to genome instability, with the intent of improving diagnosis and treatment for breast cancer. Based on our extensive preliminary data, which define HEF1 and p130Cas as novel factors controling centrosomal dynamics and spindle formation, we are exploring HEF1 and p130Cas status as contributing factors to acquisition of a malignant phenotype and drug resistance in breast cancer.

Background.

The Cas proteins are central components of integrin-dependent signaling networks. There are three defined Cas family members, including p130Cas, HEF1, and Efs/Sin. These proteins play an important role in transmission of survival signals initiated by cellular adhesion to the extra-cellular matrix mediated through integrins, at structures termed focal adhesions (1). P130Cas is ubiquitously expressed. HEF1 is present in many cell lines, but is most abundant in epithelial cells of the breast and lung, and in aggressive breast and lung carcinoma cell lines (2). In our early studies of HEF1, we had noted that the protein re-localizes from focal adhesions to the mitotic apparatus during the M phase of cell cycle (2), but the significance of this localization change, if any, had not been clear. Now we have preliminary data, which in sum leads us to the novel hypothesis that the Cas family proteins may impact tumor aggressiveness at the level of genome stability.

Aims of our study are: 1- To determinate what isoform of HEF1protein is present in the mitotic apparatus during mitosis. We will evaluate if processing by caspases is responsible for the HEF1 cleavage during mitosis and/or apoptosis. 2-To delineate the precise HEF1 sequence motifs responsible for delivery of the protein to the centrosome and to the mitotic spindle. 3-To investigate how phosphorylation of HEF1 on GSK3 kinase sites are impacted on the localization of the HEF1 protein to the mitotic apparatus. 4-To clarify the relation between HEF1 and p130Cas in the regulation centrosomal dynamic in tumor and normal breast epithelial cells.

Results

i. HEF1 localizes to the centrosome. Using high resolution confocal analysis, and two distinct antibodies specific for HEF1 in immunofluorescence, we have now determined that a population of endogenous HEF1 associates with the centrosome throughout cell cycle in MCF7 breast adenocarcinoma cells, MCF-12, MCF-10A and Hela (Figure 1). As a separate control, we have determined that overexpressed GFP-HEF1 also localizes to the centrosome, as well as to well-established intracellular destinations for HEF1 such as the focal adhesions (Figure 2). At mitosis, HEF1 spreads out from the centrosome to associate with the mitotic aster.

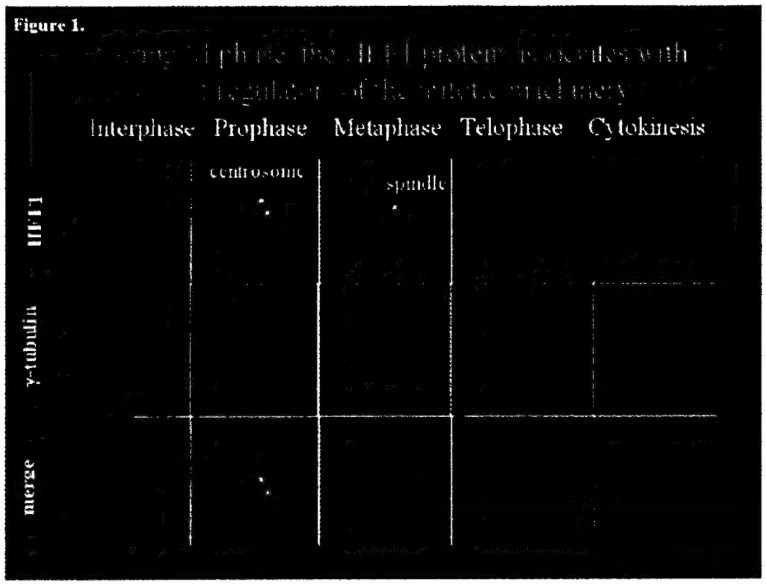


Figure 1. Immunofluorescence of cells progressing from interphase through different stages of mitosis, visualized with antibody to HEF1 (top line) or the centrosomal marker gamma-tubulin (second line): bottom line, merge of images.

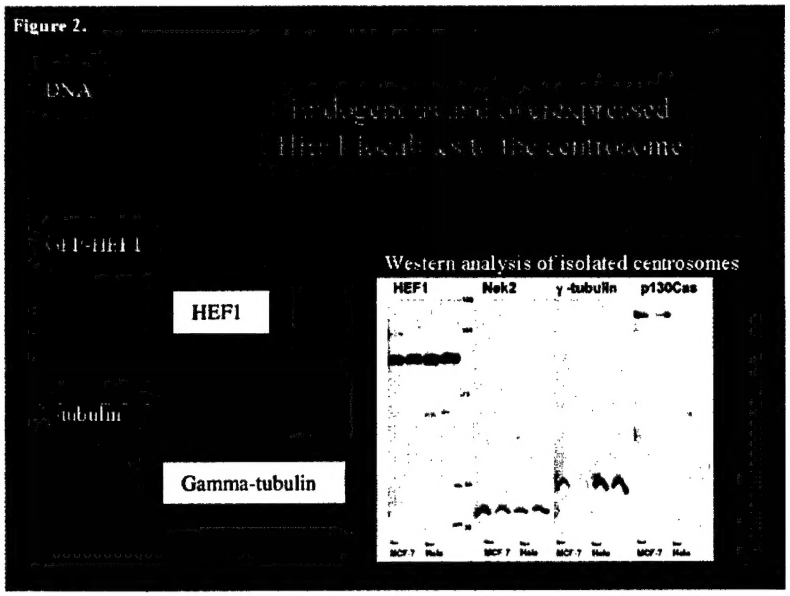


Figure 2. Left, overexpressed HEF1-fused to GFP localizes to centrosomes, based on immunofluorescence detection of co-localization with gamma-tubulin staining. Right, Western analysis of purified centrosomal preparations from MCF7 or HeLa cells, either synchronized at mitosis (Noc) or in interphase (lanes to the right of Noc lanes), using antibodies to HEF1, NEK2, gamma-tubulin, or p130Cas.

ii. Manipulation of the levels of HEF1 or p130Cas leads to profound changes in centrosomal integrity and dynamics. We have used three different approaches to manipulate HEF1 levels. The first approach is overexpression of HEF1, either by transient transfection or through use of a set of stable, tetracycline-repressible cell lines in an MCF-7 parental background (reagents described in (3)). The second approach is through use of a set of specific peptide "aptamers" selected to stabilize HEF1 in mitosis, based on our

prior observation that the protein is subject to proteolytic cleavage at this time ((2); peptide reagents described in (4)). The third approach is through use of small interfering RNAs designed to target HEF1 or p130Cas, to deplete these proteins in MCF-7 cells expressing GFP-centrin. For each class of manipulation, an appropriate matching negative control set is used.

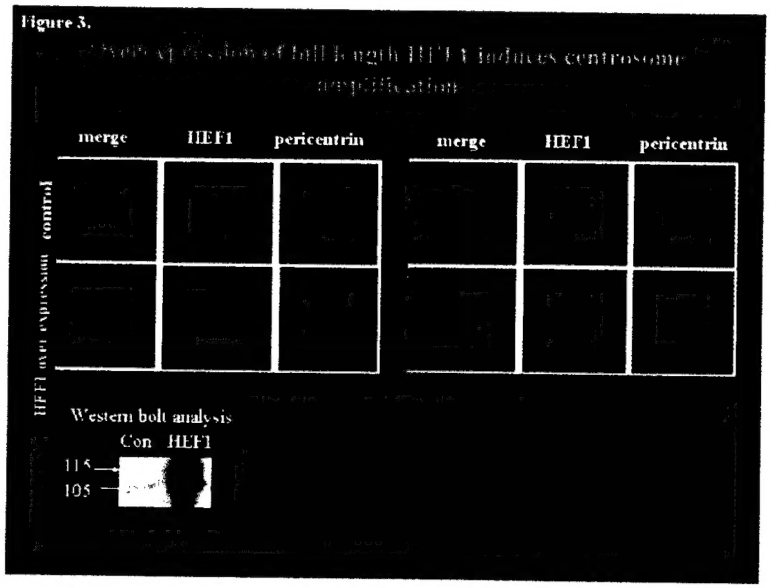


Figure 3. Spindle and centrosome defects with excess full length HEF1 in mitosis. Top panels: Use of immunofluorescence to visualize super-numerary centrosomes (left set, 6 panels) and multipolar spindles (right set, 6 panels) in cells with upregulated full length HEF1 versus controls: for each set of 6, left, merge of center, HEF1, and right, pericentrin. Bottom, demonstration of HEF1 upregulation in analyzed cells.

Each of these manipulations results in striking phenotypic changes in the centrosome. Overexpression of HEF1, or stabilization of endogenous HEF1 at mitosis with targeted peptides, in each case results in centrosomal amplification, with cells containing in excess of 4 centrosomes (detected by immunofluorescence with antibodies to gamma-tubulin) within 48 hours following treatment (Figure 3). In contrast, depletion of HEF1 (confirmed by Western analysis) resulted in a separate centrosomal defect: the premature splitting of the centrosomes in 70% of the cell population (Figure 4). Depletion of p130Cas also resulted in centrosomal defects: however, in contrast to the HEF1 depletion-dependent splitting phenotype, centrosome in p130Cas-depleted cells appeared diffuse and abnormally shaped. Intriguingly, the two phenotypes associated with over- versus under-expressed HEF1 are very similar to the recently reported phenotypes for under- versus over-expressed Cdc14A. Cdc14A is a human phosphatase that has been shown to be a crucial, centrosome-associated regulator of cell division, which when deregulated can induce genomic instability (5).

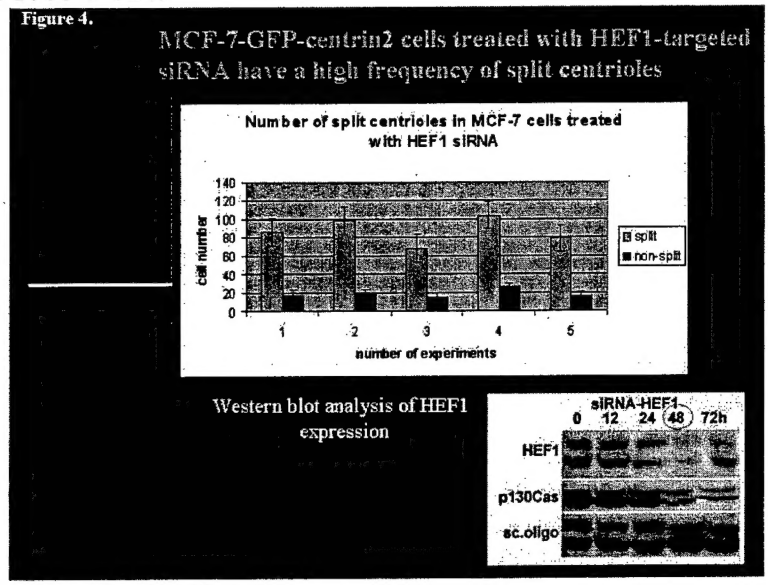


Figure 4. siRNA-depletion of HEF1 causes splitting of centrosomes. Left,. Phenotype of split centrosomes in HEF1-depleted MCF7 cells with integrated GFP-centrin: centrosomes appear as points. Right, Quantitation of splitting for cells treated with HEF1 siRNA. Bottom, demonstration that treatment with siRNA to HEF1 but not siRNA to p130Cas or scrambled siRNA leads to depletion of HEF1 from 24-48 hours following treatment· Western analysis with antibody to HEF1

iii. Phosphorylation of HEF1 is an important event in cell cycle progression.

p130Cas and HEF1 are phosphorylated by focal adhesion kinase (FAK) during cell adhesion (7), and subsequently targeted by Src family kinases in this process (6). The result of p130Cas/HEF1-FAK-Src interactions is massive tyrosine phosphorylation of Cas proteins in their SH2-binding site-rich region, nucleating complex formation with adaptor proteins such as CrkII, C3G and Sos. These phosphorylations and complex assemblies are observed in the attachment of normal cells to substrate, and strikingly enhanced following cellular transformation by oncogenes including Src and Abl. HEF1 undergoes phosphorylation changes during cell cycle progression through mitosis (results not shown), but the mechanisms and functional importance of these changes have not been investigated. We are trying to explore the function of HEF1 in mitosis, as phosphorylation of HEF1 became an important part of controlling mechanism governing HEF1 localization and interaction with target proteins. We have identified potential phosphorylation sites on HEF1 for cdc2/cyclin B and GSK3, kinases that play pivotal roles in mitotic progression. We are currently investigating the significance of interactions of HEF1 with these kinases for the observed phenotypes induced by HEF1 on the spindle and centrosomes.

***B-iii-*. Proline 47 is important in mediating p53-dependent apoptosis**

Trainee: Xiaoxian Li, Ph.D., MD
Mentor: Maureen Murphy, Ph.D.
Period report: 1/1/2003-7/1/2003

Introduction:

Breast cancer is the most common malignancy in the female and more than 1,000,000 people are diagnosed every year worldwide (8). Currently, the molecular mechanisms underlying breast cancer formation and development is not completely understood. Research results have clearly associated the initiation, development and prognosis of breast cancer with different types of genetic alterations of oncogenes and tumor suppressor genes. Among all the altered genes, the p53 gene has the highest frequency of genetic mutations. The mutation of the p53 gene is found in approximately 30% of breast cancer and is often accompanied by loss the wild type allele. Additionally, it has been shown that germline mutation of the p53 gene occurs in individuals with Li-Fraumeni Syndrome (LFS); these individuals are strongly predisposed to the development of breast carcinoma (9). Analogously, p53 null-mice typically develop thymic lymphoma; however, when thymic lymphoma development is inhibited, up to 62% of these mice develop breast carcinoma (10). Another example of the importance of p53 in the initiation of breast cancer is that several studies have indicated that mutation of the p53 gene is found in ductal carcinoma in situ before the development of invasive breast cancer (11-13). Furthermore, the frequency of the p53 gene mutation has been found to be greater in invasive breast cancer (14). Along these lines, accumulating evidence shows that mutations of the p53 gene is an independent risk factor conferring worse overall survival and some studies indicate the mutations of the p53 gene is the single most significant prognosis indicator for breast cancer (15).

Hormone therapy, chemo- and radio-therapy are the most used clinical regimens in the treatment of breast cancer. However some patients are more resistant to these clinical treatments and show worse prognosis and higher rate of relapse. Bern et al (16) show that patients with mutated p53 have the poorest response to chemotherapy, including tamoxifen. Estrogen receptor (ER) clearly involved in the etiology of breast cancer. ER positive breast cancer cells grow more slowly, are better differentiated and tend to have better prognosis. The ER has been shown to interact with and protect the p53 protein from MDM2 mediated degradation (17). The finding the ER prolongs the half-life (and activity) of p53 at least partially explains why ER positive breast cancer is typically less malignant. The combined data support a critical role for the p53 tumor suppressor in the etiology, progression and treatment of breast cancer.

p53 is a tumor suppressor protein. Mutation or deletion of p53 is found in about 50% of cancers. p53 homozygous knock out mice are prone to development of multiple types of cancer. Research shows that over-expression of p53 is sufficient to kill cancer cells. p53 suppresses tumor cell development by inducing apoptosis and arresting cells in G1 and G2 phase. At least one mechanism whereby p53 induces apoptosis and growth arrest in tumor cells is via transactivation of the expression of genes whose protein products are important in apoptosis and cell cycle. Alternatively, it has also been shown that p53 can induce apoptosis by localizing to the mitochondria of cells, and inducing cytochrome c release (18). Both of these p53-dependent apoptosis pathways require the amino terminus of p53; the amino terminal 90 amino acids of p53 are subject to extensive post-translational modification, particularly phosphorylation.

Under physiological conditions, the p53 protein level is maintained in extremely low levels, due to binding and ubiquitination of p53 by MDM2, its E3 ubiquitin ligase, which targets p53 for degradation via the 26S proteasome (see for example 19). Phosphorylation of the N-terminal of p53 repels MDM2 from interacting with p53, leading to accumulation of p53 in the nucleus. Furthermore, phosphorylation

of the N-terminal increases the interaction between p53 with co-activators, such as p300, increasing the transcriptional activity of p53 (20). p300 can acetylate promoter-associated histones and make DNA more accessible to transcriptional regulators.

Serine 46 (S46) is a newly identified phosphorylation site on the N-terminus of p53; importantly, phosphorylation of Serine 46 is known to be essential for efficient p53-dependent apoptosis. Mutation of S46 to an alanine residue decreases the ability of p53 to induce apoptosis up to 5-fold (21and Li and Murphy, unpublished observations). At least part of the mechanism whereby phosphorylation of Serine 46 confers apoptosis by p53 is that this modification increases the ability of p53 to transactivate the pro-apoptotic p53-response gene p53AIP1 (21). Current understanding of the phosphorylation process of S46 is that several protein kinases, including the inducible MAP-kinase family member p38, are the kinases that phosphorylate S46 of p53 (21). p38 is a proline-directed kinase, and Serine 46 of p53 is directly followed by a proline residue. This proline residue would be predicted to be critical for the ability of p38 to phosphorylate p53. We are studying a rare polymorphism of p53 that exists in up to 5% of African Americans, that changes the proline residue at amino acid 47 to Serine. We hypothesize that this polymorphism, S47, would inhibit the ability of p53 to be phosphorylated by p38, and decrease the apoptotic ability of p53. A corollary to this hypothesis is that individuals with the S47 polymorphic variant would have increased risk of cancer, including the cancer that is predominant in Li-Fraumeni patients, breast cancer.

Our results show that the Serine 47 polymorphism (S47) has impaired ability to induce apoptosis, compared to wild type p53. We show that this variant has greatly reduced ability to be phosphorylated at Serine 46, along with decreased ability to induce apoptosis. We are currently testing the hypothesis that the S47 polymorphic variant has decreased apoptotic ability because it has decreased ability to transactivate the p53-response gene p53AIP1 (which requires phosphorylation of Serine 46).

Body

i-Generation of stably-transfected cell lines containing wt p53, the Serine 47 polymorphic variant (SS) and a point mutation that eliminates Serine 46 (AP)

We generated two point mutation p53 constructs, one encoding the Serine 47 variant encoding S46S47 (SS) and one in which the Serine 46 phosphorylation site is eliminated A46P47 (AP). These, and wild type p53 (wt) are all of human origin and were cloned into CMV-neo-BAM3 using site-directed mutagenesis (Stratagene). The correct sequence of the plasmids was confirmed by sequence analysis of the entire p53 cDNA. H1299 and SAOS-2, which are both p53-null tumor cell lines were transfected with the plasmids and stable cell lines were sub-cloned following selection in G418. The sub-cloned cell lines were shown to have equal level of p53 by western analysis (see westerns in Figure 7). In order to generate inducible clones of p53, we used temperature sensitive (TS) p53 with a point mutation of codon 138. At 39°C, this p53 protein is mis-folded and does not bind to DNA or show transcriptional activity. However, when shifted to 32°C, the protein is properly folded and shows wild type transcriptional activity. Cell lines were maintained at 39°C to inactivate p53. During experiments, cells were shifted to 32°C to activate p53, typically for 24 hours.

ii-The S47 polymorphic variant shows altered phosphorylation of S46.

Because the phosphorylation of S46 has been shown to be important for the apoptotic function of p53, we examined whether the S47 variant has altered phosphorylation (in other words, whether P47 is required for the phosphorylation of S46). An antibody that specifically recognizes p53 only when it is phosphorylated at S46 was used to detect the phosphorylation of S46.

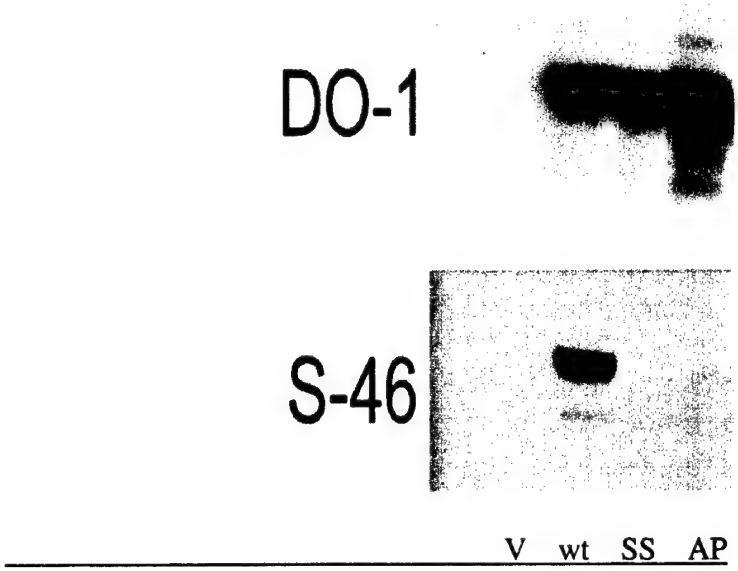


Figure 6. Proline at amino acid 47 is important in the phosphorylation of Serine 46 (S46) of p53. *H1299 cells were transiently transfected with empty vector, wt, SS and AP p53 plasmids. The cells were shifted to 32°C 24 hrs after transfection and harvested and lysed 24 hours after temperature shift using NP-40 buffer supplemented with protease inhibitors. Cell lysates were loaded on an SDS-PAGE gel and subjected to Western Analysis. DO-1 is a monoclonal antibody against the N-terminus of p53 and detects all forms of p53. S-46 denotes the phospho-S46 specific antibody, which only detects p53 with phosphorylated S46. AP, the negative control, can not be phosphorylated on codon 46.*

As expected, SS showed dramatically decreased reactivity to the antibody (Fig. 6), indicating compromised phosphorylation of S46. D0-1 antibody recognizes all forms of p53, and serves as the positive control.

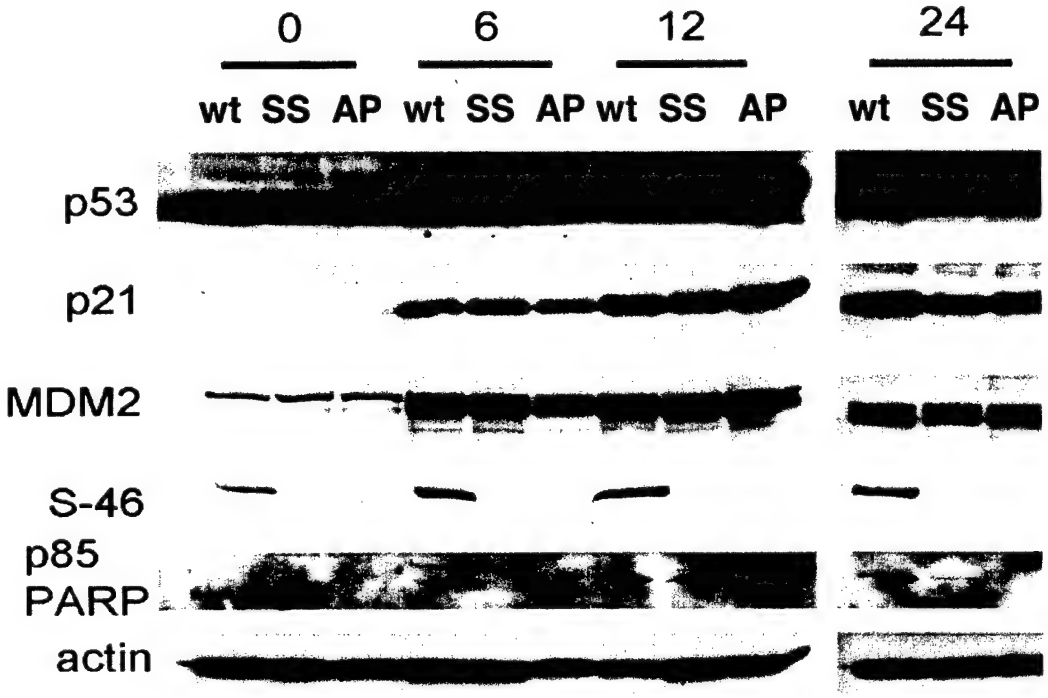


Figure 7. Wild type (wt) p53 has greater apoptotic potential than SS and AP after temperature shift. Stable H1299 cells expressing wt, SS and AP p53 were shifted to 32°C and harvested at indicated time points after temperature shift. Cell lysates were loaded on a SDS-PAGE gel and subjected to Western analysis. Equal amount of protein was loaded confirmed by the level of actin. P85-PARP is the caspase-cleaved form of PARP, which is cleaved during apoptosis. MDM2 and p21 are two known transcriptional targets of p53.

iii-Wild type (wt) p53 has greater apoptotic potential than SS (Serine 47) and AP (alanine 46)

We next examined whether the altered phosphorylation pattern of S46 changed the apoptotic function of p53. Our results show that wt p53 is more apoptotic than SS and AP after temperature shift, and that the Serine 47 (SS) form of p53 actually shows intermediate apoptotic function, compared to wild type and AP, in stably-transfected H1299 cell lines (Figs. 7 and 8). The difference of apoptosis appeared at 6 hrs after temperature shift and reached the maximum at 24 hrs. Importantly, no apoptosis was observed at 0 hr (39°C), meaning the apoptosis is p53-dependent. The phosphorylation of S46 can be observed at 39°C but significantly increased after temperature shift, and the increased phosphorylation of S46 preceded the apoptotic events. These observations indicate that the phosphorylation of S46 is important in the apoptotic function of p53 and the codon 47 regulates this phosphorylation and the apoptotic ability of p53. Interestingly, non-apoptotic genes, such as p21 and MDM2, were up-regulated by wt, SS and AP p53 to the same level (Fig. 7), indicating that wt p53 may specifically up-regulate certain apoptotic genes, like p53AIP1, differently from the Serine 47 variant, but this may not be true for all p53-response genes.

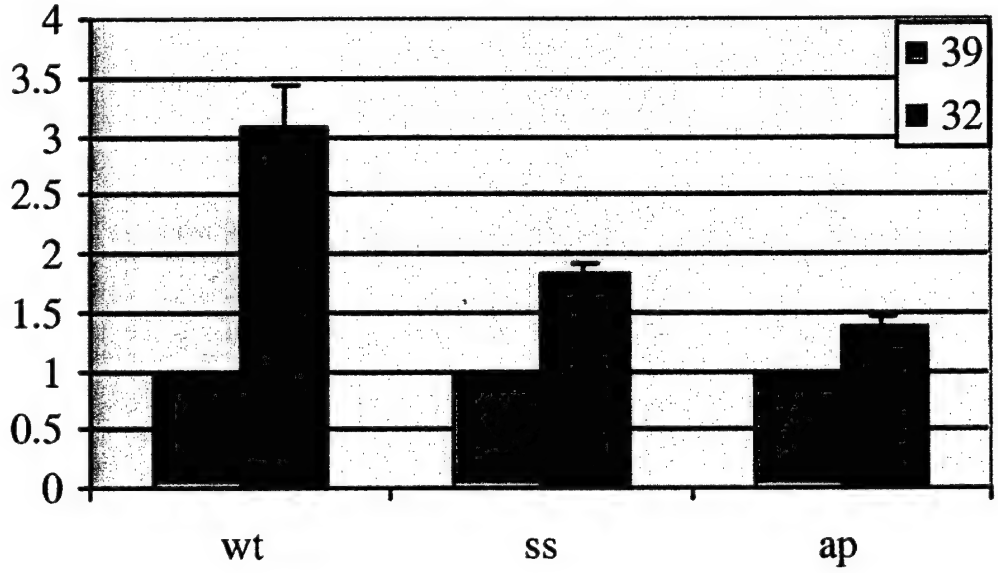


Figure 8. Multi-caspase assay confirms the greater apoptotic function of wt p53, compared to the Serine 47 polymorphic variant (SS) and the negative control, AP. H1299 cells were treated the same as described in Fig. 2. The apoptotic cells were detected by detecting the pan-caspase activity using the Guava flow cytometer (Guava Technology). The number of apoptotic cells at 32°C is normalized to that at 39°C. Wild type p53 is significantly more apoptotic than SS and AP ($P<0.05$), but SS and AP are not significantly different in apoptotic ability. The results come from experiments conducted on two independent H1299 cell lines of each construct. The bars are presented as mean±SE (4 experiments).

iv-Wild type p53 is more apoptotic after ionizing radiation than the Serine 47 polymorphic variant

Our data have shown that wt p53 is more apoptotic after temperature shift in inducible cell lines. We next asked the question whether this was also true in response to DNA damage. We first treated the H1299 cells with ionizing radiation (IR). Figure 9 shows that the difference in apoptosis appeared 6 hrs after treatment and reached the maximum at 24 hrs. Importantly, the difference in apoptosis at 24 hrs is

dramatically more than that when cells were just treated with temperature shift (compare Fig. 7 and 9). Here apoptosis is measured by the appearance of the 85 kD form of PARP, which is generated by caspase activation.

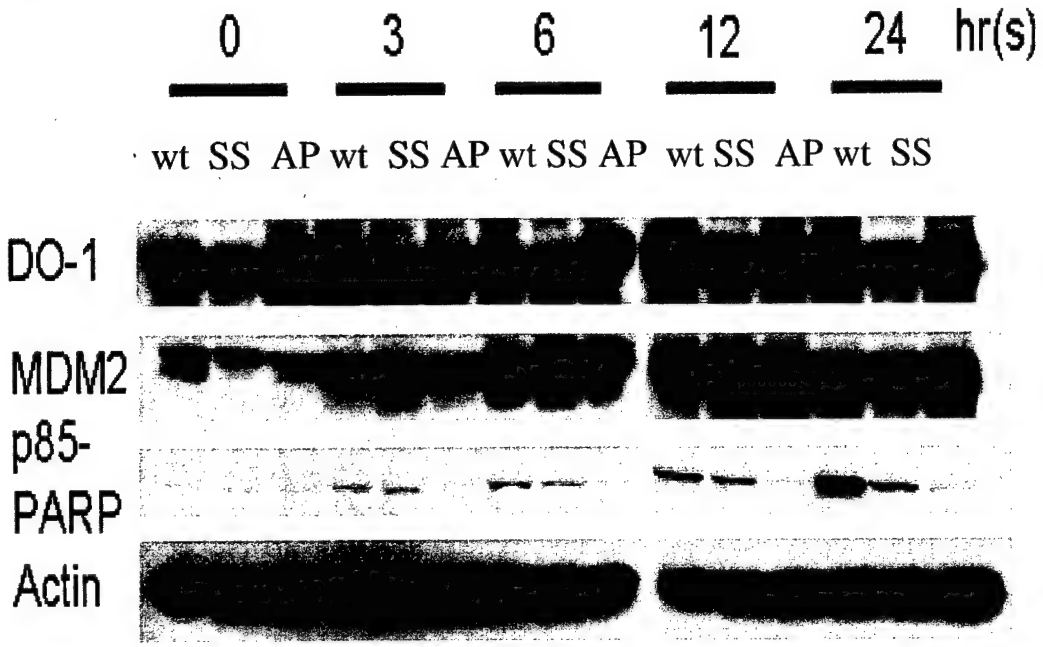


Figure 9. Wild type p53 demonstrates greater apoptotic ability than the Serine 47 polymorphic variant (SS)or the negative control (AP) following treatment with ionizing radiation (IR). Stably transfected H1299 cells were treated with IR (7.5 Gy) and shifted to 32°C right after IR treatment. Cells were harvested at indicated time points. Cell lysates were loaded on an SDS-PAGE gel and subjected to Western analysis with the indicated antibodies.

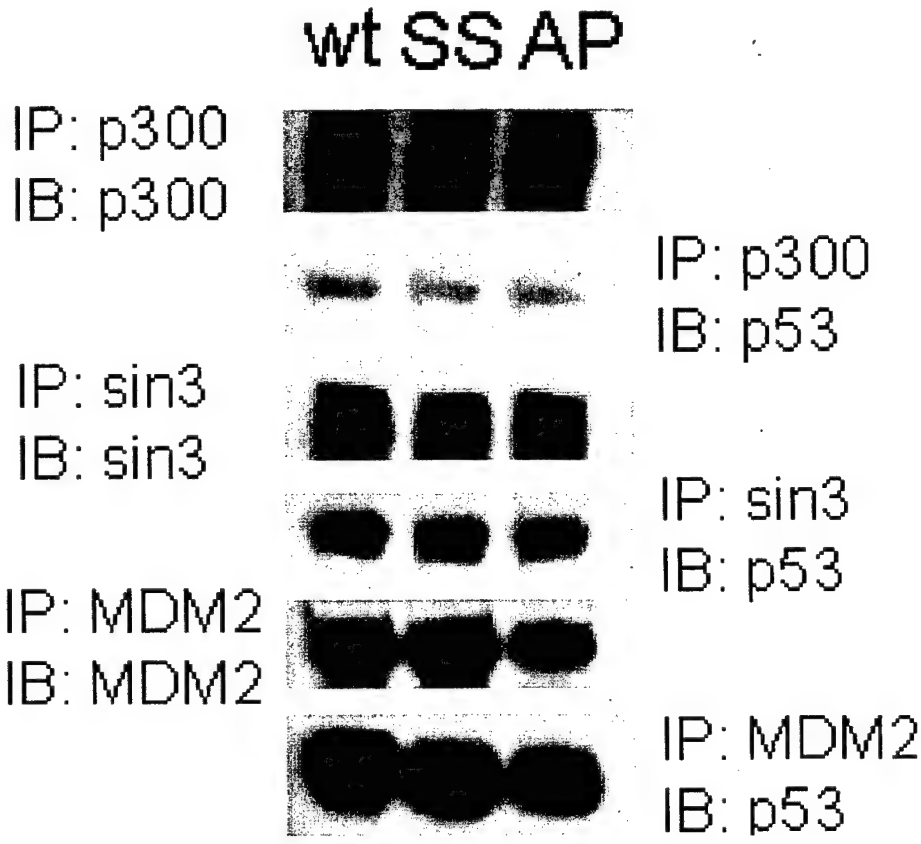


Figure 10 Wild type p53 binds to p300, MDM2 and Sin3 in a manner indistinguishable from SS and AP. Stably transfected H1299 cells were shifted to 32°C and harvested 24 hrs after temperature shift. 2000 ug of protein and antibody against p300, MDM2 or sin3 was incubated at 4°C overnight after which protein A sepharose was added and incubated for 30 minutes at room temperature. The immuno-complex was washed twice with NP-40 buffer followed by 2 washes with RIPA buffer. The immuno-complex was then loaded on an SDS-PAGE gel and subjected to Western analysis.

v-*The apoptotic difference between wt and SS p53 is not cause by different binding ability to p300*

Given the compelling evidence showing that the wt p53 is more apoptotic than SS and AP, we tried to find out the molecular mechanism behind the difference. p300 can acetylate core histones and make DNA more accessible to transcriptional regulators (22).

Reports have shown that p300 can bind to p53 as a co-activator and acetylate the C-terminus of p53 to increase its transcriptional activity (23). We immunoprecipitated p300 and asked whether the wt, SS and AP proteins bound to p300 the same. Our results show that the binding ability to p300 is the same between wt and SS and AP p53 (Fig. 5), confirmed by the same level of acetylation on codon 373 (data not shown), which is specifically acetylated by p300 (23). We also tested whether two other p53 bound proteins, MDM2 and Sin3, bound to wt, SS and AP the same. Again, our results show there is no difference in the ability of these p53-binding proteins to bind to the wild type, AP and Serine 47 variants (Fig. 10).

p53-null mice develop breast cancer (23a). Additionally, women with Li Fraumeni syndrome, which have germline mutations in p53, develop breast cancer as their most common tumor type. Therefore, it will be important to correlate our findings with breast cancer. In this regard we have made great efforts to generate stably-transfected lines of wild type p53, the Serine 47 variant and the AP negative control in the background of the cell line PN6, which is a p53-null transformed mammary epithelial cell line (24). Analysis of these cell lines will determine if the S47 polymorphic impacts breast cancer; all expectations are that it will. An added bonus to this study is that the impact of p53 induction on breast carcinoma cell lines (that is, apoptosis versus differentiation versus growth arrest) has never been formally studied, so the wild type cell line should also prove useful to analyze.

DAMD1700-1-0249 RUSSO, Jose

***B-iv.*- Development of a Mouse Model for the Targeted Disruption of the *Appl* Gene in Mammary Gland**

Trainee: Huihong You, Ph.D
Mentor: Joseph Testa, Ph.D.
Period report: 1/1/2003-7/1/2003

Introduction

Like other AKT/protein kinase B (PKB) family members, AKT2 is activated by various growth factors through phosphatidylinositol 3-kinase (PI3K) and thereby mediates signals involved in diverse cellular processes, including apoptosis inhibition, cell proliferation, and insulin signaling. AKT is an integral player in a signal transduction pathway of which many components have been linked to oncogenesis. Activated forms of AKT and its upstream activator, PI3K, are responsible for the transforming activities of certain viruses, and a negative regulator of this pathway, PTEN, is a tumor suppressor.

To facilitate our understanding of the cellular function of AKT2, we had previously used the yeast two-hybrid system to identify two candidate AKT2 partners. Multiple independent isolates of two clones were obtained in a screen of a human fetal brain cDNA library. We have designated one of the putative AKT2 interactors APPL (25). Although a number of important substrates and regulators of AKT/PKB have been identified, our data indicate that APPL is not a substrate but, instead, is an adaptor. To date, APPL is the only adaptor reported to interact with AKT kinases. Moreover, under the conditions tested thus far, this interaction has been strongest with the AKT2 isoform. Since AKT2 appears to play a more prominent role in human neoplasia than other members of the AKT family, the characterization of APPL may provide important insights regarding oncogenic mechanisms involving AKT2. Thus, the characterization of APPL has been a priority of our laboratory.

We previously mapped the *APPL* gene to human chromosome 3p14.3-p21.1, a site of frequent deletions in a variety of tumor types. However, we screened a large number of tumor cell lines exhibiting LOH in the 3p region and did not observe marked down-regulation of APPL expression, suggesting that APPL is required for cell viability.

Recently, the APPL adaptor protein has also been shown to form a complex with Rac or Cdc42. APPL siRNA has been shown to induce apoptosis, although it is not known at this time whether this effect is due to inhibition of Akt's pro-survival function or to an effect on Rac-related MAPK signaling. In any case, our data suggest that APPL is an essential cellular protein that plays a significant role in cell proliferation and cell survival.

Apoptosis occurs during normal growth and development of the mammary gland. During involution and remodeling of the breast after lactation, apoptosis is a prominent feature. Most of the secretary epithelium in the lactating breast undergoes apoptosis as the mammary gland regresses and is reorganized for another cycle of lactation. The expression of activated Akt is regulated in this process, such that Akt1 activation peaks in pregnancy and lactation, and decreases during mammary involution (reviewed in reference 26). To provide insights regarding how the loss of *Appl* regulates Akt activation and the normal apoptotic process, and how loss of *Appl* affects mammary development during lactation and involution, we plan to generate an *Appl* conditional knock-out (KO) mouse model. Since Akt2 and Her2 overexpression or *Pten* deletion play important roles in mammary gland development and mammary tumorigenesis, we propose to elucidate the biochemical significance of Appl in these processes. To address these questions, we intend to cross *Appl* KO mice with MMTV-*Akt2* or MMTV-*Her2* transgenic mice, or with *Pten* KO mice.

Body

To date, we have finished Specific Aim 1. This Aim includes: screen phage libraries derived from 129Sv mouse genomic DNA to identify phage clones containing the *Appl* coding region; and construct a plasmid for gene-targeted disruption of APPL.

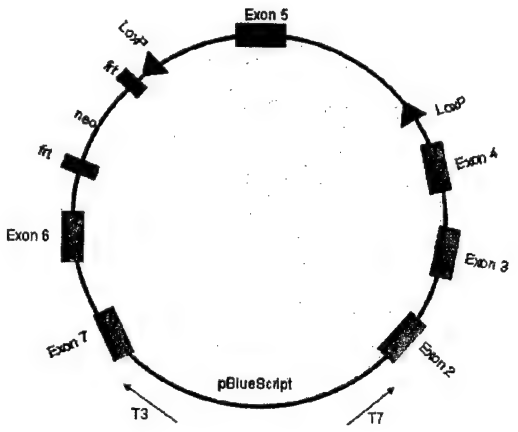
i- A phage 129Sv mouse genomic library (Stratagene) was screened and clones containing the Appl coding region were identified

Based on the human *APPL* genomic sequence and restriction mapping, we decided to target Exon 5 of the mouse *Appl* gene. We generated a probe specific for Exon 5 of the mouse *Appl* gene, based on mouse EST sequences, and used this probe to screen the library. We identified 5 positive clones, which are about 15.5 kb in length and contain *Appl* genomic sequences that flank exon 2 to 7 or exon 5 to 11.

ii- A construct to conditionally disrupt Appl expression/function was synthesized

The *Appl* gene has 22 exons distributed over ~60 kb. Exon 5 is 88 bp in length and is flanked by two very large introns, which maximize the chance that our engineered cassette will recombine specifically with the *Appl* locus. Disruption of Exon 5 will result in a frame shift in the *Appl* ORF, which is predicted to disrupt endogenous *Appl* gene transcription.

Figure 11: Knockout targeting construct for disruption of Exon 5 of *Appl*. Processing of the *Appl* ORF will be disrupted after Exon 4.



We devised a detailed restriction map of the region of interest. With this information, a targeting vector was designed to add one LoxP site upstream of Exon 5 and another LoxP and a selectable neomycin-resistance gene (*neo*) cassette flanked by two FRT sites downstream of Exon 5. Unique restriction fragments have been identified that can distinguish between the wild-type and the LoxP/*neo-Appl* genes by Southern blot and PCR analyses.

Overlapping subclones obtained from the phage genomic library encompass approximately 15.5 kb of sequence. Figure 11 depicts the complete *Appl* KO construct that has been constructed for the investigations described. In brief, the 9.3-kb genomic sequence flanking Exon 2 to Exon 7 of mouse *Appl* has been directly excised by restriction enzyme from a subclone derived from a 129Sv mouse genomic library. This 9.3-kb insert was cloned into a pBluescript vector (with mutated KpnI site, XbaI site, SpeI site and BamHI site in its multiple cloning region). An oligo with a LoxP sequence and an EcoRI site has been cloned into the BamHI site of the vector upstream of Exon 5. Another LoxP site with FRT-flanked neo sequence derived from plasmid pK-11/pM-30 (27) has been cloned into the vector downstream of Exon 5, between EcoRV and KpnI sites.

We will electroporate ES cells with this KO construct and screen the resultant transfected clones for homologous recombination of the gene-targeted construct using Southern blot analysis and PCR and electroporate positively identified gene-targeted ES cells with plasmids, which can express Flp site-specific recombinase to remove the neo selection cassette.

After deriving a homologous recombined ES clone, we will generate mice for future KO studies and breed chimeric mice to obtain mice heterozygous and homozygous for LoxP incorporated *Appl* alleles. We will breed these mice with MMTV-Cre or WAP-Cre transgenic mice to disrupt the *Appl* gene in mammary gland, delineate the phenotype of *Appl*-targeted disruption in the mammary gland, and elucidate the function of Appl in breast development and during mammary involution. These studies may provide insights regarding *in vivo* mammary specific apoptotic signaling and potential targets for therapeutic intervention.

We also intend to breed floxed *Appl* mice to EIIa-Cre transgenic mice to generate mice with *Appl* deleted during embryogenesis. If disrupting the *Appl* gene during embryogenesis is not lethal, we will breed these mice with MMTV-*Akt2* transgenic mice, with *Akt2* KO mice (previously generated by Dr. D. Altomare in our laboratory) to further delineate the role of Appl in mammary gland development, or with *Pten* KO mice (available from our collaborator, Dr. A. Cristofano) or with MMTV-Her2 transgenic mice to study the function of Appl in mammary tumorigenesis. Previous data from our laboratory showed that Akt plays a critical role in mammary gland involution. By breeding *Appl* KO mice with MMTV-*Akt2* transgenic mice or with *Akt2* KO mice, we will determine if Appl can regulate Akt function in normal mammary gland development, especially during lactation and involution. About 60% of *Pten*-deficient mice develop breast cancer by 10 months (A. Di Cristofano, personal communication). By breeding *Pten* KO mice or MMTV-*Her2* transgenic mice with *Appl* KO mice, we will be able to investigate whether Appl deficiency can decrease the incidence, size, or aggressiveness of mammary tumors.

In order to unravel the full spectrum of *Appl* function, it is important to analyze this gene in the context of a living animal. The use of *Appl* conditional KO mice will complement our laboratory's ongoing *in vitro* studies. Gene inactivation through homologous recombination is an unambiguous means to target *Appl*, thereby eliminating its function and establishing a phenotype for Appl loss-of-function. The resulting *Appl* KO mice will be interbred with WAP-Cre or MMTV-Cre transgenic mice, EIIa-Cre transgenic mice, and other transgenic or knockout mice with genetic defects in the Akt signaling pathway to determine if Appl has an important physiological function in mammary gland development and mammary tumorigenesis.

B-v- Cloning of a new gene/s in chromosome 17p13.2-13.1 that control apoptosis

Trainee: Sandra Fernandez, Ph.D
Mentor: Jose Russo, MD
Period report: 1/1/2003-7/1/2003

Introduction

Breast cancer is a hormone dependent malignancy whose incidence is steadily increasing in most western societies and in countries that are becoming industrialized (28-32). Despite significant advances in early detection, surgery, radiation, chemo- and endocrine therapy, the mortality caused by this disease, that in the United States is only second to lung cancer as a cause of cancer-related death, has remained almost unchanged for the past five decades (28). New studies of the biochemical mechanisms evoked by conventional treatments for neoplastic diseases point to apoptosis as a key process for elimination of unwanted cells (33). Impaired function of apoptosis-related genes is deeply involved in oncogenesis and the progression of cancers (33-36). Among the genes that control apoptosis is Fas (CD95/Apo-1) a cell membrane receptor that upon binding by its ligand (FasL) triggers a signal resulting in apoptotic cell death (35-37). Fas is a cell-surface receptor that exists in two forms, transmembrane and soluble. The former induces apoptosis by ligation of FasL or agonistic anti-Fas antibody, whereas the latter inhibits Fas-mediated apoptosis by neutralizing its ligand (38, 39). Harnessing the power of this complex molecule is expected to lead to the development of powerful chemotherapeutic advances. Our Laboratory has recently found a link between the Fas ligand-receptor complex in chemically transformed human breast epithelial cells and a gene/s located in chromosome 17p13.2p13.1 (40). Whereas, gene transfer of Fas ligand (CD95L) using adenoviral vectors has been shown to generate apoptotic responses and potent inflammatory reactions that can be used to induce the regression of malignancies *in vivo*, there are significant unwanted reactions such as hepatotoxicity that may limit their clinical utility (34, 37). Therefore what is needed is to identify which is the gene or genes that may regulate Fas ligand-receptor complex (39, 41-45). For this purpose we have proposed the following aims: **1) To isolate in the precise location in chromosome 17p13.2-p13.1 the gene(s) responsible for the control of Fas-receptor ligand complex function, 2) To test the cloned gene/s functionality in cell transformation by cDNA transfection studies in vitro, and 3) To determine the functional role of the isolated gene in the process of neoplastic progression in vivo**.

Body

i--The experimental system. We have developed an *in vitro - in vivo* system in which the environmental carcinogen benz(a)pyrene (BP) has been utilized for inducing *in vitro* the transformation of human breast epithelial cells (HBEC) (46-59). For developing this paradigm of human breast cancer we have capitalized in the availability of the mortal HBEC-MCF-I0M, which without viral infection, cellular oncogene transfection, or exposure to carcinogens or radiation became spontaneously immortalized, originating the cell line MCF-I0F (60, 61). Treatment of MCF-10M cells with chemical carcinogens failed in inducing cell transformation, while MCF-10F cells responded to *in vitro* treatment with BP with the expression, in a progressive fashion, of all the phenotypes indicative of neoplastic transformation. BP-treated MCF- I0F cells expressed increased survival and formation of colonies in agar methocel, loss of ductulogenic properties in collagen matrix, invasiveness in a Matrigel *in vitro* system (clones BP-l) and tumorigenesis in severe combined immunodeficient (SCID) mice (BPI-E) (46, 49, 54). We have demonstrated, by utilizing DNA amplification of microsatellite length polymorphism, allelic losses and microsatellite instability in different areas of the genome of the HBEC transformed by chemical carcinogens (51, 52, 61-66). MSI was detected in chromosomes 11 and 17 in the immortalized cells, and additional MSI in chromosomes 11, 13 and 17 in the transformed cells. LOH was detected only in one

locus of chromosome 17p13.2-p13.1 in the transformed cells BPI-E using the marker D17S796 (40) (Figure 12). We have been a unable to identify any other genetic alterations in more than 40 other markers, including p53, in the p or q arm of chromosome 17 (40). Because chromosomes 11 and 17 were involved in both the early and late stages of carcinogenesis we selected them for testing their functional roles in chemically transformed HBEC using a microcell-mediated chromosome transfer (MMCT) technique 62-67). Our study found that seven out of ten clones with chromosome 17 transferred in to BP1E cells had reverted transformed phenotypes such as advantageous in growth, colony formation in agar-methocel, loss of ductulogenesis and resistant to Fas mediated apoptosis (40). Our molecular analysis of MCF10F and BP1E cells reveled that in the process of chemical transformation loss of genetic material (LOH) in the chromosome 17 p13.2 (D17S796), which has been identified by other investigators in atypical ductal hyperplasia and in situ ductal carcinoma of the breast (68), has been restored in the cells with abrogated transformation phenotype (Figure 12).

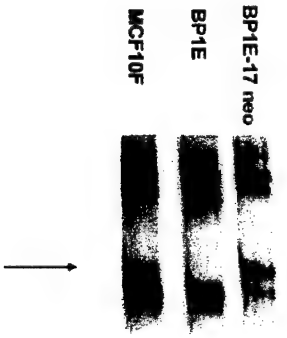


Figure 12: LOH in BP1E using the marker D17S796 and restoration of this region in BP1E-17 neo. Single-stranded conformation polymorphism (SSCP) analyses were done using DNA from MCF-10F (line 1), BP1E (line 2) and BP1E-17 neo clone II-3 (line 3). PCR reactions were performed in a total volume of 10 µl containing: 1X buffer with $MgCl_2$ (Perkin Elmer), 400 µM of each dATP, dTTP, dCTP and dGTP, 200 µM of the primer D17S796 Reverse, 200 µM of the primer D17S796 Forward, 0.2 U Taq Polymerase (Perkin Elmer), 1 µCi [^{32}P-α] dATP and 120 ng total DNA. The thermal cycling consisted on: 94°C, 2 min for denaturalization, and 35 cycles of 94°C, 45 sec; 56°C, 45 sec and 72°C, 45 sec followed by an extension at 72°C, 10 min. PCR products were run on 6% polyacrylamide gel and autoradiographied.

This indicates that the LOH in this region may regulate the early event of breast cancer initiation and that the region of D17S796 (7586bp) might contains gene/s controlling the transformation phenotypes. More importantly it was observed that in those clones in which the reversions of the phenotype were present also the cells, loss the resistance to Fas mediated apoptosis. Transfer of chromosome 11 neither revert the transformation phenotypes or the resistance to the Fas mediated apoptosis. These data allowed us to postulate that chromosome 17 p13.2 (marker D17S96) might contain a gene or genes that are controlling this process.

-ii-a- Methods and procedures.

DNA isolation: DNA was prepared from MCF-10F, BP1E and BP1E-17 neo (clone II-3). All the cells cultures were treated with lysis buffer (100 mM NaCl, 20mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS) with 200 µg/ml proteinase K and incubated at 65°C, 15 minutes with gentle agitation. The samples were cooled down on ice and treated with 100µg/ml RNAse at 37°C, 30 minutes. The samples were purified by extracting with 1 volume phenol followed by an extraction with 1 volume of chloroform: isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75M with ammonium acetate and the DNA

was precipitated with 2 volumes of 100% ethanol. The samples were centrifuged, dried and dissolved in distilled water.

PCR amplification: PCR amplification was performed using the High Fidelity PCR system (Roche). The primers used to amplify were: Primer JR 853 Reverse 5' CTCTCTTAGGATTTACCTTCCC 3' and Primer JR 864 Forward 5' TCTTTCTGCTGATGGCTCTCC 3'. The genomic DNA from MCF-10F, BP1E and BP1E-17 neo (clone II-3) were use as templates in different PCR reactions. The PCR was performed in a 100μl volume containing 100 ng of genomic DNA, 1X buffer with $MgCl_2$, 200 μM of each dATP, dCTP, dGTP, and dTTP, 300 nM of each primer, and 5U Expand High Fidelity enzyme (Roche). The PCR conditions were: denaturalization at 94°C, 2 min followed by 30 cycles at 94°C for 15 sec, 60°C for 30 sec and 72°C for 50 sec, followed for 72°C, 10 minutes for extension. The 10 minutes extension is important to ensure that all PCR products are full length and 3'adenylated (A).

Cloning of the PCR products: The PCR products were cloned in the vector pCR2.1 using the TOPO TA cloning kit (Invitrogen). The linearized vector supplied has a single, overhanging 3' ends deoxythymidine (T) residues. This allows PCR inserts 3' adenylated to ligate efficiently with the vector. The ligations were used to transform *Escherichia coli* TOP10 (Invitrogen) using the chemical transformation method. The transformants were plated on LB agar with 50μg/ml kanamycin and X-gal and the plates were incubated at 37°C during 24h. The plasmid pCR 2.1 has a fragment of the *lac*Z gene and the cloning site is within this region. The peptide LacZ produces a blue color when the bacteria are plate on X-Gal. The LacZ peptide will not be present and the colonies remains white if the *lac*Z gene has been interrupted by the cloned insert.

Plasmid preparation and Sequencing: Minipreparations of the plasmid DNA were made using the alkaline lysis method. The pellet was resuspended in 100 μl of distilled water and treated with RNAase (20 μg/ml) during 30 minutes at 37°C. One volume of PEG/NaCl (13% 6-8K PEG, 1.6M NaCl) was added and incubated on ice 30 minutes. The samples were centrifuged 30 minutes at 4°C. The pellet was resuspended in 50 μl of distilled water. The sequencing reactions were done at the Fox Chase Cancer Center Facility. The M13 Reverse and M13 Forward primers were used for sequencing. The Sequencher and GCG programs were use for the sequences analysis.

RT-PCR: RNA was extracted from MCF-10F, BP1E and BP1E-17neo using Trizol reagent (Life Technologies, Inc.) and the pellet was resuspended in DEPC water. Approximately, 4 μg of RNA was treated with 4 U DNase I (Ambion) during 30min at 37°C followed by the addition of 5 μl of Dnase Inactivation reagent (Ambion) according to manufacture protocol. Another step of DNase I inactivation was made by incubation at 80°C, 10 minutes. The sample was divided in two tubes with 2 μg of RNA each. One of these RNA samples was used to prepared cDNA and the other was used to test contamination with DNA (negative control). For the cDNA synthesis, 2 μg of RNA was mix with 0.4 μg oligo $(dT)_{12-18}$ in a final volume of 9.6 μl and incubated at 70°C during 10 min and cool down on ice. The following reagents were added: 3.2 μl 5X First Strand buffer, 0.8 μl 10mM dNTP and 1.6 μl 0.1M DTT. After incubation during 5 min at 42°C, 160U SuperScript II Reverse Transcriptase (Invitrogen) was added. The reaction mix was incubated at 42°C, 1 h. The enzyme was inactivated at 70°C, 15 min. The reaction mix was cool down on ice and 1.6U of *E. coli* RNase H was added and incubated at 37°C, 15 min. After the cDNA synthesis, the PCR reactions were done.

The primers JR869 Reverse and JR870 Forward were used and a PCR fragment of 340 bp was expected. In other PCR reactions, the primers JR 867 Reverse and JR 868 Forward were used and the expected product was 273-285 bp. The conditions for both PCR were the same. The PCR was performed in a 50 μl volume containing 50 ng of DNA, 1X buffer with 1.5 Mm $MgCl_2$, 200 μM of each dATP, dCTP, dGTP, and dTTP, 200 nM of each primer, and 5 U of Platinum Taq (Invitrogen). The PCR conditions

were: denaturalization at 94°C, 4 min and 35 cycles at 94°C for 45 sec, 56°C for 45 sec and 72°C for 45 sec, following an extension at 72°C, 10 minutes. The sequences of the primers are: Primer JR 869 Reverse: 5' CTG CTC CAG GAT TTC AAG GAC C 3', Primer JR870 Forward: 5' GCT GAT GGC TCT CCT ATG ACC 3', Primer 867 Reverse: 5' CTT GGG GTA TAC ATG CAC CTG 3' and Primer 868 Forward: 5' TCT CCC TGT GAG CAT AGG GTT GAC 3'. RNA treated with DNase I (without reverse transcription) was used in the PCR reactions to test for DNA contamination (negative controls).

ii-b- Results.

Cloning of D17S796 region.

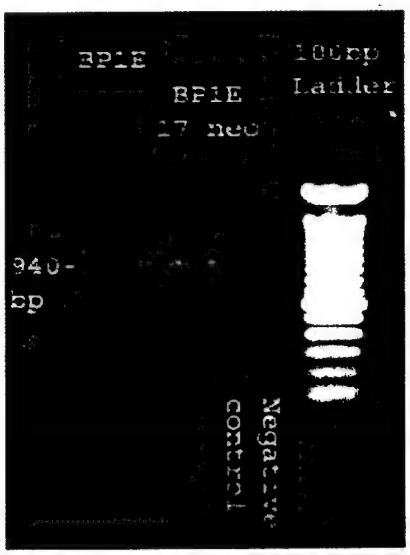
It has been reported that the frequent LOH of a specific chromosomal marker is indicative of a closely linked tumor suppressor gene (TSG) (69,70). In previous experiments we have shown LOH in the locus 17p13.2 using the microsatellite marker D17S796 in BP1E cells (Figure 12), and a detailed analysis of this region using Map Viewer (http:// www.ncbi.nlm.nih.gov/ mapview/map_search.cgi) has shown the KIAA0523 gene of approximately 200 Kb upstream and the AIPL1 (aryl hydrocarbon receptor interacting protein-like 1) at 78 Kb downstream of this marker. Therefore, we pursued the search for a novel putative TSG in this region, which is approximately 940 bp. We have amplified this region by PCR from MCF-10F, BP1E and BP1E-17 neo (clone II-3), using the primers JR 853 and JR864. The sequences of these primers were obtained based on the sequence from the Genebank AC005668 (Figure 13).

```
                                                                  JR 853 →
126421 aatgtttttt gattcaataa actctgtttt tcagctttga tgctactggt gtaactactc

       →
126481 tcttaggatt taccttccct gaggggcagc ctggtctaaa agtcactcca ttgtgcactg
126541 gatgaagggc ccaggaggtg agtgagaaca tcaaagtggg agtggggcca ggcaggtgcc
126601 actcttggca gcttgctctc cccagctctc ctaaagccag ggtcagagca tgagccccgg
126661 atgggagggg catcatcctg gacctgatca ggcctagaag agcttgcgtt ctcaaccgag
126721 gctggtcttt accaagatca tccccaacga cctgccctcc ccagccatct ttcctcccat
126781 atgctacacc ctacatgtct tgcaaagctg atgtcccctg tggctccttg actgttcatg
126841 tctctctgac ctctcctttc tcagaccatg ggtatgtgag cagctaggat caaggggcat
                                                          D17S796 Rev
                                                                    →
126901 gagatttagt cctgacgaca tgatcattcc atgtgtgaat ctggtttgcc cctttagtcc

       →
126961 gataatgcca ggatgtgcct ctttcacagt agcctttatg ctctcctgag tacttggggt
127021 atacatgcac ctgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtta aaaaatactg
127081 ctccaggatt tcaaggacca catttggttc cattgttttc tttaattgtg gcaagctaag
                  ←
                        D17S796 Forw
127141 aagaaaagaa ctgcaatgcg catgctctgt agatggacat gcttcccatg gtcattacac
127201 cagaggagag tcgggcacag ctcctccccc tgccaggctc ccaagatcca tgctccaatg
127261 tcaaccctat gctcacaggg agagccttga tttaagtttg gtaaaacgat atcccattaa
127321 atttatgttg ttaatttgaa ttttattgat ttcgttgttt gccgttctat aatttgtcca
127381 tttgttttgc ttttatggtc ataggagagc catcagcaga aagattttac tccagcttgc
                                    ←                          JR864
127441 atgttggtac atatttaaat agcattataa tagaaattag ttgtgtcaac aaagagggag
127501 aagttctttg aaagtatttt catttcaaaa gaagagccaa gcattgctag gatttgagaa
127561 atattttatt taatgttgta tttcccacga gaggatcatg agcgctcaac tcttcccaac
```

```
127621 cccagctgac tcctccatga gaaatctgtc agtggttttc ccagaaggct gcccccgtgt
127681 gaaattcaaa gatatgtagt gtgttaaggc cagggtgttg ctctccgaat gcctccacca
127741 ggaagaatct gggacccagc ttctgcccag ttacaaatga atgggacaaa gtcatgttca
127801 ctggaaactt gcctgagaaa ccatctgagg acgtcattgt gcgtccatcg ttccgagaaa
127861 acatgagctg tgaaagacca caggacagat gcagaggact cccctaaatt atctacccca
127921 gggagtgctg gcatttctct ctatccctga tgccagcaaa tgcagccacg cccaagagat
127981 tttttctttc tttgtccccc acgagaagaa gatatggaga ctatgtggtt attaaagctc
128041 ttagc 128046
```

Figure 13: Nucleotide sequence of the region D17S796 from the GeneBank AC005668. Part of the nucleotide sequence of the GeneBank AC005668 (Homo sapiens chromosome 17, clone hRPK.243-K-12), bases126421 to 127440, is shown. The position of the primers D17S796 Reverse and D17S796 Forward used in the SSCP analyses are underline. Also, the position of the primers JR853 and JR864 used in the PCR amplification are shown.

The 940bp region was amplified from using a high fidelity Taq polymerase (Figure 14). The PCR products were cloned in the vector pCR2.1 and the mix was used to transform *E. coli* TOP 10 (Invitrogen). The plates were incubated at 37°C during 24 h for detecting the growth of blue and white colonies (Figure 15). Ten white colonies were picked up and grown on LB with kanamycin.



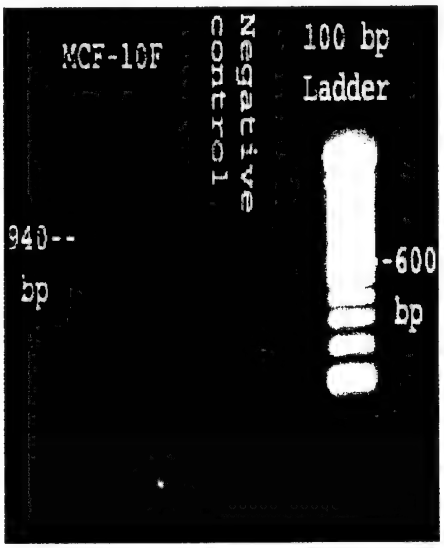


Figure 14: PCR products obtained from MCF-10F, BP1E and BP1E-17 neo clone II-3. The primers JR853 and JR864 were used to amplified 940 bp region contained D17S796. The total DNA from MCF10-F, BP1E and BP1E-17 neo (clone II-3) were used as template in three different PCR reactions. Approximately, 10 µl of each PCR reaction was run on 1.5% agarose gel. The 940 PCR products are shown. PCR reactions without DNA were also made as control (negative control). A 100 bp ladder was run in both gels as molecular weigh marker.

Figure 15: White and blue colonies growth on LB agar with kanamycin and X-gal. The PCR products obtained were cloned in the vector pCR2.1. The plasmids were used to transform E. coli TOP 10. White and blue colonies growth after 24h incubation at 37°C. This plate shown the colonies obtained using the PCR product amplified from BP1E. White colonies harbored plasmids with the 940bp insert. Blue colonies harbored pCR2.1 without insert. Ten white colonies were picked up for further analyses.

Sequence analyses.

The plasmids were prepared using the alkaline lysis method followed by PEG/NaCl precipitation. The inserts were sequenced at the Fox Chase Cancer Facility using M13 Reverse and Forward primers. The sequences from the different clones were compared using Sequencher and GCG programs. We found differences between MCF-10F, BP1E-17 neo and BP1E in a region of 160-174 bp that corresponds to the marker D17S796 that contains a zone of TG repetition. Using the multiple sequence alignment, we found that the sequences from BP1E differ with MCF-10F and BP1E-17neo basically in the number of TG repetition that lies between the primers D17S796 Reverse and D17S796 Forward (Figure 16). In the Figure 16, the alignment between the sequences from BP1E and MCF-10F/BP1E-17neo are shown. Approximately, 530 bp that include D17S796 region is shown. The only missing bases were found in this region.

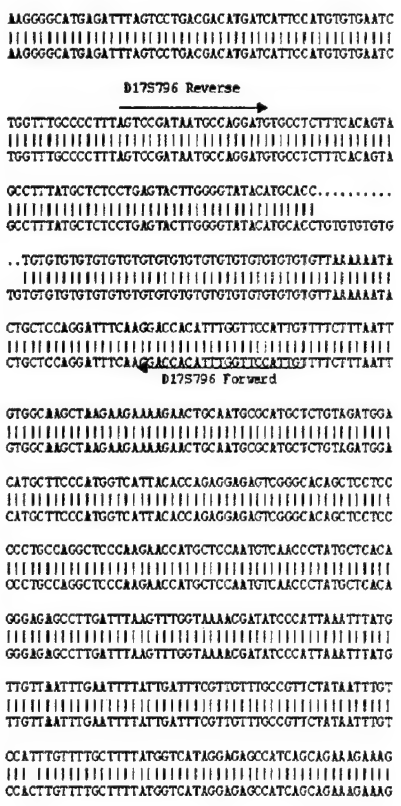


Figure 16: Sequences alignment between Bp1E and MCF-10F and BP1E-17 neo. Approximately, 550bp nucleotide sequence from BP1E with 19 TG repetitions is shown on the top. The nucleotide sequence from MCF-10F and BP1E-17neo with 25 TG repetitions is shown at the bottom. The positions of the primers D17S796 Reverse and D17S796 Forward are underline.

Sequences with different number of TG repetitions were found in the same cell line. In MCF-10F, five alleles were found (Figure 17). One colony from 9 (1/9) shown 18 TG repetition; three colonies (3/9) shown 20 TG repetitions; one colony (1/9) shown 21 TG repetition; two colonies (2/9) shown 24 and two others (2/9) shown 25 TG repetitions (Figure 6). In BP1E, two different alleles were found (Figure 17). Five out of 7 colonies analyzed (5/7) shown 20 TG repetitions and two (2/7) shown 19 TG repetitions (Figure 6). In BP1E-17 neo, five alleles were found (Figure 17). Two out of 10 analyzed colonies (2/10) shown 19 TG repetitions and 2 (2/10) shown 20 TG repetitions. Three colonies out of 10 analyzed (3/10) showed 22 TG repetitions, one (1/10) shown 23 and two (2/10) shown 25 TG repetitions (Figure 17).

Our PCR-SSCP analysis of MCF10F and BP1E cells reveled that in the process of chemical transformation loss of genetic material (LOH) in the chromosome 17 p13.2-p13.1 (D17S796), has been restored in the cells with abrogated transformation phenotype. The LOH found in BP1E using PCR-SSCP (Figure 12) is explained by the lost of the bigger PCR products. The expected sizes of the PCR

products using D17S796 Reverse and D17S796 Forward are indicated in each case based on the sequences obtained (Figure 17). According to our previous results, one of these fragments, present in MCF-10F and BP1E-17 neo and absent in BP1 could be responsible for the reversion of the transformed phenotype of BP1E-17 neo. We found that BP1E has one allele with $(TG)_{19}$ and the other allele with $(TG)_{20}$ (Figure 17). The same fragments were also found in BP1E-17 neo. From the others three alleles present in BP1E-17 neo, only the one with 25 TG repetitions was also found in MCF-10F. It is possible that this fragment was responsible for the reversion of the phenotype of BP1E-17 neo.

		Number of colonies	Expected size of the D17S796 fragment
MCF-10F:			
	.....CACC $(TG)_{16}$ TT AAAAAA.....	1/9	160 bp
	.....CACC $(TG)_{20}$ TT AAAAAA.....	3/9	164 bp
	.....CACC $(TG)_{21}$ TT AAAAAA.....	1/9	166 bp
	.....CACC $(TG)_{24}$ TT AAAAAA.....	2/9	172 bp
	.....CACC $(TG)_{25}$ TT AAAAAA.....	2/9	174 bp
BP1E:			
	.....CACC $(TG)_{19}$ TT AAAAAA.....	2/7	162 bp
	.....CACC $(TG)_{20}$ TT AAAAAA.....	5/7	164 bp
BPE1-17 neo:			
	.....CACC $(TG)_{19}$ TT AAAAAA.....	2/10	162 bp
	.....CACC $(TG)_{20}$ TT AAAAAA.....	2/10	164 bp
	.....CACC $(TG)_{22}$ TT AAAAAA.....	3/10	168 bp
	.....CACC $(TG)_{23}$ TT AAAAAA.....	1/10	170 bp
	.....CACC $(TG)_{25}$ TT AAAAAA.....	2/10	174 bp

Figure 17: Differences inMCF10F, BP1E and BP1E-17 neo in the different clones. There are different numbers of TG repetitions in the D17S796 region. Five different alleles are found in MCF-10F and BP1E-17 neo, and, two alleles in BP1E. The number of colonies with the corresponding insert and the total number of colonies analyzed in each case are indicated in the first column. The expected size of the PCR fragments using the primers D17S796 Rev and D17S796 Forward are indicated at the right (bp: base pairs).

We have performed a detailed computational analysis of the fragment with 25 TG repetitions isolated from MCF-10F and BP1E-17 neo. Sequence translation shown a putative peptide of 131 amino acids (Figure 18). The predicted amino acid sequence does not seem to share significant homology with any known protein.

```
1    TTTAGTCCGA TAATGCCAGG ATGTGCCTCT TTCACAGTAG CCTTTATGCT CTCCTGAGTA
       .  S  D  N  A  R  M  C  L  F  H  S  S  L  Y  A  L  L  S
61   CTTGGGGTAT ACATGCACCT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT
     T  W  G  I  H  A  P  V  C  V  C  V  C  V  C  V  C  V  C  V
121  GTGTGTGTGT TAAAAAATAC TGCTCCAGGA TTTCAAGGAC CACATTTGGT TCCATTGTTT
     C  V  C  V  K  K  Y  C  S  R  I  S  R  T  T  F  G  S  I  V
181  TCTTTAATTG TGGCAAGCTA AGAAGAAAAG AACTGCAATG CGCATGCTCT GTAGATGGAC
     F  F  N  C  G  K  L  R  R  K  E  L  Q  C  A  C  S  V  D  G
241  ATGCTTCCCA TGGTCATTAC ACCAGAGGAG AGTCGGGCAC AGCTCCTCCC CCTGCCAGGC
     H  A  S  H  G  H  Y  T  R  G  E  S  G  T  A  P  P  P  A  R
301  TCCCAAGAAC CATGCTCCAA TGTCAACCCT ATGCTCACAG GGAGAGCCTT GATTTAAGTT
     L  P  R  T  M  L  Q  C  Q  P  Y  A  H  R  E  S  L  D  L  S
361  TGGTAAAACG ATATCCCATT AAATTTATGT TGTTAATTTG AATTTTATTG ATT
     L  V  K  R  Y  P  I  K  F  M  L  L  I  .
```

Figure 18: Translation of the sequence present in MCF-10F and BP1E-17 neo. The putative peptide of 131 amino acids obtained from the sequence analyzes is shown. The first codon of this putative exon (TCC) that encodes the amino acid serine (S) is underlined.

Comparison of the DNA sequences of many different genes revealed certain similarities at the intron-exon junctions (71). The base sequence of the intron usually ends with AG. The

3' splice site sequence of most introns is similar to the sequence $(C/U)_{<10}N(C/T)AG/G$ such that most introns end in AG after a long stretch of pyrimidines (53). These sites appear to be very important for introns processing. We have found that in the sequence of this novel protein, the bases AG are present at the 3' putative splice site (Figure 18). In mammalian genes, polyadenylation sites are usually preceded by AATAAA or ATTAAA ~20 bases before the cleavage site and followed by a more weakly conserved GT-based motif. A putative polyadenylation site ATTAAA and GT region was found at the 3' end. These data suggest that this peptide could be the last exon of a novel protein. This peptide forms part of a larger novel protein and it is possible that also others part of this protein would be mutated in BP1E.

Sequences analyzes from BP1E, showed that translation of the sequence with 19 TG repetitions will give a putative peptide of 127 amino acids similar to the one found in BP1E and BP1E-17 neo (Figure 19). These peptides differ in four amino acids: VCVC (V: valine; Cysteine). The translation of the sequence with 20 TG repetitions, also found in BP1E, have a non-sense codon (stop codon) and a shorter peptide of 61 aminoacids.

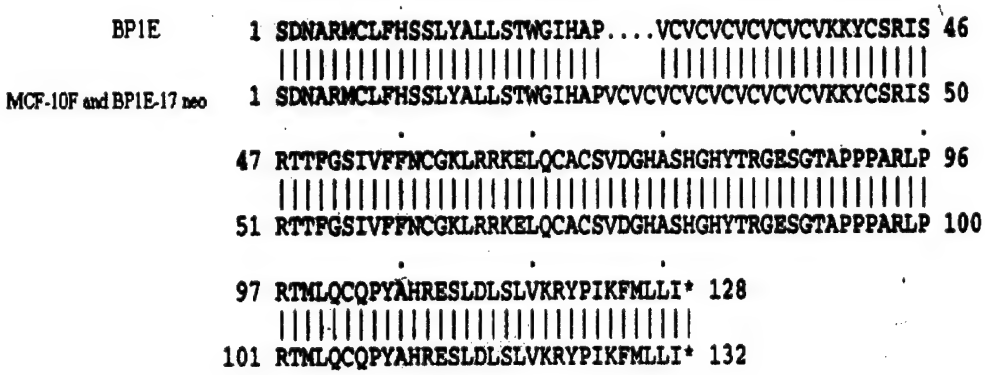


Figure 19: Alignment between the putative peptides from BP1E and the putative peptide from MCF-10F and BP1E-17 neo. The peptide alignment has shown that four aminoacids are missing in BP1E.

The putative peptide was compared with known proteins using BLASTP. The predicted peptide was compared with a known proteins using BLASTP. The Swiss-Prot/ TrEMBL primary accession numbers are shown (http://us.expasy.org/sprot). The putative peptide of 131 amino acids showed very low homology with known proteins (Table 1). The nuclear transition protein 2 (DNA-binding protein) shown 22.1% similarity and 15.3% identity.

Table 1. Homology between the predicted peptide of 131 amino acids and different known proteins.

Protein	% Identity	% Similarity	Accession numbers
Transitional protein 2 (Nuclear transition protein 2)	15.3%	22.1%	Q9N1A7
Similar to expressed sequence AW049250	14.5 %	22.1%	Q8K116
Histamine H3 receptor (HH3R)	14.5%	22.1%	P58406
Netrin 4 (β netrin)	11.5%	17.6%	Q9JI33

Study of the expression of the region that contains D17S79.

As our "*in silico*" analyzes has shown a putative novel peptide in the region D17S796. The next step was to use RT-PCR to determine if there is expression of this region in MCF-10F, BP1E and BP1E-17neo cells. For this purpose total RNA was extracted from these cells and cDNA was synthesized using reverse transcriptase and oligo(dT). The cDNA obtained from the different cell lines were used in PCR reactions with specific primers for the putative coding region. Primers with annealing sites within the putative coding region were used in the amplifications and PCR products would be expected only if there is mRNA corresponding to this region (Figure 20). A fragment of 340 bp was obtained in the three cell lines when the primers JR869 and JR 870 were used (Figure 21). A fragment of approximately 285 bp was obtained when the primers JR867 and JR868 were used (Figure 21). These results showed that the region that harbors D17S796 is transcribed to mRNA in MCF-10F, BP1E and BP1E-17 neo.

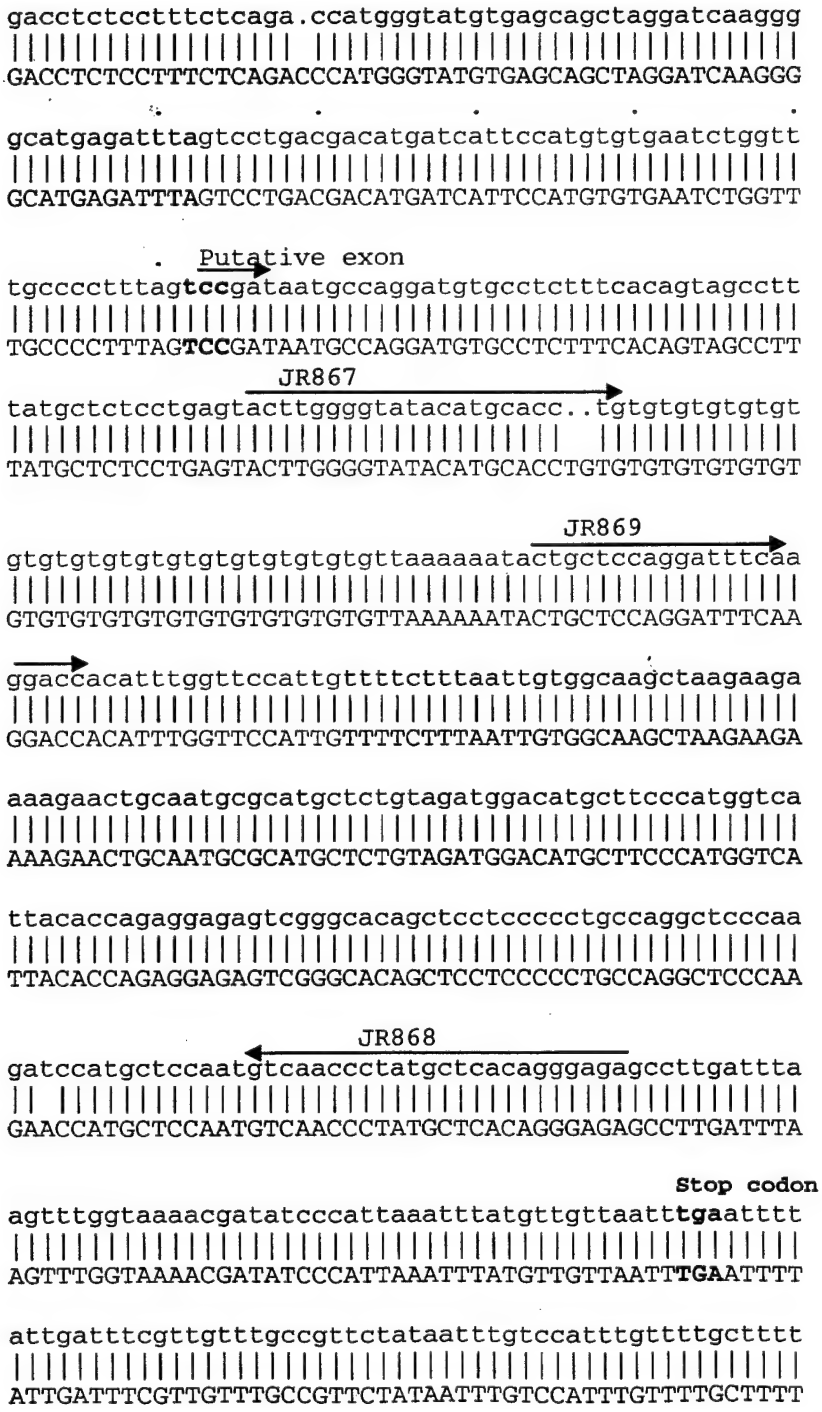


Figure 20: Annealing sites of the primers used in RT-PCR experiments. The sequences of AC005668 (upper sequence) and MCF-10F (bottom sequence) are shown with the corresponding sites for the primers used in the RT-PCR: JR869, JR870, JR867 and JR868. Also the site for JR864 is shown. The beginning of the putative exon, the stop codon and the ATTAAA at the 3' are shown.

JR864

atggtcataggagagccatcagcagaaagattttactccagcttgcatgttg

ATGGTCATAGGAGAGCCATCAGCAGAAAGA

JR870

gtacatatttaaatagcattataatagaaattagttgtgtcaacaaagagggagaagttctttgaaagtattttcatttcaaaagaag
agccaagcattgctaggatttgagaaatattttatttaatgttgtatttcccacgagaggatcatgagcgctcaactcttcccaaccccagctg
actcctccatgagaaatctgtcagtggttttcccagaaggctgcccccgtgtgaaattcaaagatatgtagtgtgttaaggccagggtgttgct
ctccgaatgcctccaccaggaagaatctgggacccagcttctgcccagttacaaatgaatgggacaaagtcatgttcactggaaacttgcctga
gaaaccatctgaggacgtcattgtgcgtccatcgttccgagaaaacatgagctgtgaaagaccacaggacagatgcagaggactcccctaaatt
atctaccccagggagtgctggcatttctctctatccctgatgccagcaaatgcagccacgcccaagagatttttctttctttgtcccctacga
gaagaagatatggagactatgtggtt **attaaa**gctcttagc

Also we found that the expressed-sequence tag EST 3179739 matches a region that is located 120 bp downstream of the cloned region (bases 127541 to 127544). The EST 3179739 sequence comes from a cDNA library from lung (tissue type: carcinoid). Expressed sequence tags (ESTs) are short (200-500bp) DNA sequences generated from the 3' and 5' ends of randomly selected cDNA clones and the purpose of EST sequencing is to rapidly scan for all the protein coding genes and to provide a tag for each gene on the genome. A 99% identity was found between both sequences using Blast (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

These results strongly suggest that we have cloned the last exon of a novel protein.

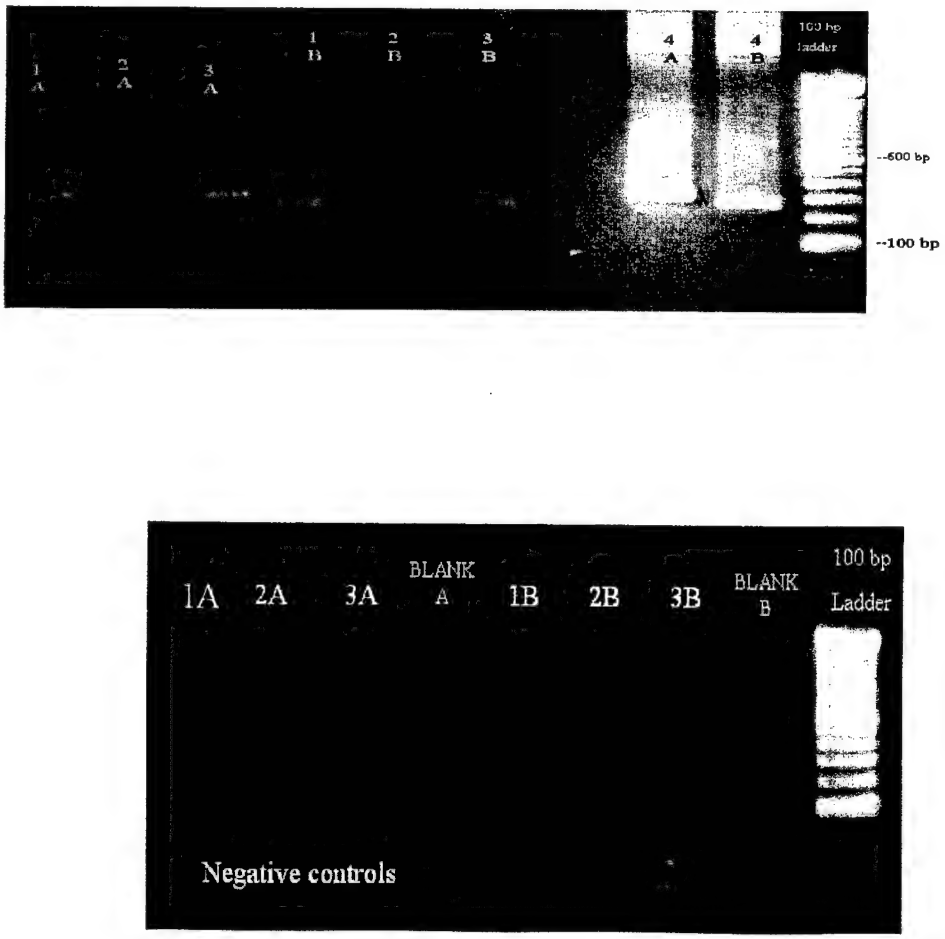


Figure 21: Study of the expression of the D17S796 region in MCF-10F, BP1E and BP1E-17 neo by RT-PCR experiments. Upper gel: RNA from MCF-10F (1), BP1E (2) and BP1E-17neo (3) were used for reverse transcription. The cDNA obtained were used in PCR reactions. The primers JR 869 and JR870 were use in some reactions (A). In another PCR reaction, the primers JR867 and JR868 were used (B). Fragments of 340bp and 285 bp were obtained respectively. Bottom gel: RNAs from the different cells were used directly in the PCR reactions as control of DNA contamination without reverse transcription. Also, reagents controls for both PCR reactions were made (Blank A and Blank B). A 100bp ladder was run at the right.

C- KEY RESEARCH ACCOMPLISHMENTS.

C-i- It has long been appreciated that cells undergo profound changes in physical organization during mitosis. Although an extensive literature has developed in the analysis of cytoskeletally-associated signaling proteins in interphase cells, it is only recently that the possible role of these proteins in mitosis has been appreciated. Our work on HEF1 is the first and only study that has clearly indicated an important function of the Cas proteins in this context. Defects in the integrity of cellular division at mitosis are important predisposing factors to breast cancer, as reflected by the defects in ploidy and overduplication of centrosomes in many primary breast cancers. Hence, unequivocal establishment of a critical action of cytoskeletally linked signaling proteins in the regulation of spindle function and cytokinesis would provide completely new insight into the significance of the known upregulation of many of these proteins in cancer.

C-ii- The Serine 47 polymorphic variant of p53, which occurs in up to 5% of African Americans, demonstrates decreased phosphorylation of serine 46.

The Serine 47 polymorphic variant of p53 demonstrates significantly decreased apoptotic potential than wild type p53 ($p<0.05$).

The apoptotic difference between wt, and SS and AP p53 is not caused by different binding ability to co-activators, such as p300.

C-iii- C-iii- We have isolated and characterized genomic fragments of Appl from a phage 129Sv mouse genomic library. A 9.3-kb sequence, flanking exon 2 to exon 7 of Appl, was used to generate a targeting construct containing LoxP sites and neomycin resistance sequence. We are using this homologous construct to electroporate embryonic stem cells that will be selected for resistance to G418 and homologous recombination of the targeting construct.

C-iv- We found a novel gene in the region D17S796. This gene is located at approximately 200 Kb of the KIAA0523 gene and 78 Kb of AIPL1 (aryl hydrocarbon receptor interacting protein-like 1).

We have cloned the last exon of this novel gene and the sequence of this fragment will be used to select the full- length cDNA.

RT-PCR experiments shown that this gene is expressed in MCF-10F, BP1E and BP1E-17 neo and the EST 3179739 matched the 3' end of this gene.

We found difference in the region D17S796 between MCF10F, BP1E-17 neo and BP1E. The difference is in the number of the TG repeats in this region. We found that MCF10F and BP1E-17 neo has one allele in which the D17S796 region is 174 bp although, BP1E shown a shorter region.

D- REPORTABLE OUTCOMES

1.Lareef, M.H., Tahin, Q., Russo, I.H., Mor, G., Song, J., Mihaila, D, Slater, C.M., and Russo, J. Transfer of chromosome 17(p13.1) to chemically transformed human breast epithelial cells induces Fas-mediated apoptosis. Proc. Am. Assoc. Cancer Res. 42:1475a, 2001.

2 Mello, M.L.S., Lareef, M.H., Hu, Y-F., Yang, X.Q., Vidal, B.C. and Russo, J. RNA relocation at mitosis in benz (a) pyrene transformed human breast epithelial cells after microcell mediated transfer of chromosomes 11 and 17. Proc. Am. Assoc. Cancer Res. 42:4781a, 2001

3.Lareef, M.H., Tahin, Q., Russo, I.H., Mihaila, D., Tomaz, J., Tosolini, A., Testa, J., and Russo, J. Chromosome 17(p13.1) transfer reverts transformation phenotypes in human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 42:4803a, 2001.

4.Mello, M.L.S., B.de Campos Vidal, Lareef, M.H., J. Russo. Changes in chromatin texture in transformed cells as assessed by molecular biology assays and image analysis. 14th International Congress of Cytology May 27-31 Amsterdam-The Netherlands.

5.Mello, M.L.S., Lareef, M.H., Vidal, B.C. and Russo, J. RNA relocation at mitosis in benz (a) pyrene transformed human breast epithelial cells after microcell mediated transfer of chromosomes 11 and 17. (Submitted for publication, Analytical cellular pathology, 2001).

6.Miller, S.M., Driscoll, J.L., Rodoletz, M., Sherman, K.A., Daly, M.B., Diefenbach, M.A., Buzaglo, J.S., Godwin, A.K., & Babb, J.S. (2001, January). Coping style correlates of participation in genetic testing for inherited breast and ovarian cancer risk. Poster presented at A Decade of ELSI Research Conference, January 15-17, Bethesda, MD.

7.Sherman, K.A., Mutton, N., Nour, C., Wainwright, S., & de Torres, A. (2001). Predictors of breast self-examination in young women. Rapid Communication Poster, Society of Behavioral Medicine 22nd Annual Scientific Session, March 21-24, Seattle, WA.

8.Miller, S.M., Rodoletz, M., Buzaglo, J.S., Gray, T., & Sherman, K.A. (2001). Monitoring style in low-income minority women at risk for cervical cancer: Cognitive-Social determinants of adjustment and adherence. Symposium Paper, Society of Behavioral Medicine 22nd Annual Scientific Session, March 21-24, Seattle, WA. Annals of Behavioral Medicine, 23(Suppl.), 117.

9. Miller, S.M., Sherman, K.A., Buzaglo, J.S., & Rodoletz, M. (in press). Monitoring-Blunting behavioral signatures in coping with health threats: The example of cancer. *Psicologia della Salute.*

10. UGT1A6 Genetic Polymorphisms: Identification and Genotype/ Phenotype Analysis from Human Liver Tissues." J.J. Zalatoris, PhD* and R.B. Raftogianis, PhD, Dept. Pharmacology, Fox Chase Cancer Center, Philadelphia, PA. Presentation at the 2001 Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics, Orlando, FL, March, 2001.

11. Andrey Frolov, Zhong-Zong Pan, Dominique Broccoli, Lisa Vanderveer, Nelly Auersperg1, Henry Lynch,Mary Daly, Thomas Hamilton, and Andrew K. Godwin Identification of ovarian cancer-associated genes using a HOSE cell transformation model. Ninth SPORE Investigators's Workshop, Washington, DC, July 15-17, 2001".

Reportable outcomes from January 1 to July 30, 2003:

Presentations by E. Pugacheva:

1. American Society for Cell Biology, December 14-18th, San Francisco, CA (poster)
2. Fox Chase Cancer Center Postdoc Day, 6 June 2003, Philadelphia, PA (poster)
3. The Engelhardt Institute for Molecular Biology Biennial Meeting, June 29th – July 5th 2003, Moscow, Russia (talk)

Presentation by X. Li

1-Li, X., Dumont P., and Murphy M (2003). Graduate and Postdoctoral Fellows Day at Fox Chase Cancer Center. Philadelphia, PA

Publications:

1. Serebriiskii, I.G., Mitina, O., Pugacheva, E., Benevolenskaya, E., Kotova, E., Toby, G.G., Khazak, V., Kaelin, W.G., Chernoff, J., and Golemis, E.A. Detection of peptides, proteins, and drugs that selectively interact with protein targets. Genome Res 12: 1785-1791, 2002.

E- CONCLUSIONS AND SIGNIFICANCE:

E-i- Predisposition to genomic instability in breast cancer: analysis of molecular mechanisms. The phenotypes we have observed in cells over expressed or depleted of HEF1 is exactly coincident with the cells similarly manipulated for c-NAP1. Based on preliminary date we proposed that HEF1 could be a cohesion factor in the centrosome of epithelial cells and play the same role as c-NAP1 in the fibroblast, i.e., preventing centriolar separation. In our model, overexpression of HEF1 keeps centrioles together and supports over-duplication, but depletion of HEF1 causes centriolar splitting and defects in spindle formation. The regulatory mechanism for the disassociation of HEF1 protein from the centrosome could be phosphorylation of HEF1 by the mitotic Cdc2-cycB kinase, and/or GSK3 kinase during earlier stages of cell cycle progression. Our data for the first time suggest an additional model for the function of the Cas proteins in cancer, wherein defective signaling through HEF1 or p130Cas also promotes genomic instability, allowing rapid selection for other genetic changes associated with cancer progression, including increased tendency to drug resistance.

E-ii-Our results show that the Serine 47 polymorphic variant, which eliminates proline at amino acid 47, demonstrates greatly impaired phosphorylation on Serine 46; this is consistent with the findings by others that Serine 46 is chiefly phosphorylated by proline-directed kinases, such as p38. These data were recently confirmed by in vitro kinase assays using purified p38 kinase (X. Li and M. Murphy, unpublished data). Notably, our data indicate that p53 with compromised phosphorylation of S46 is much less apoptotic than wt p53 after temperature shift and IR treatment. Our results further show that the apoptotic difference is not caused by the binding ability to co-activators such as p300. Our observations that the S47 form of p53 has impaired ability to induce apoptosis following ionizing radiation may have implications for the treatment of breast carcinoma with radiation therapy. This possibility remains to be determined. Along these lines, the Serine 47 polymorphism may alter cancer risk, and the phosphorylation level of Serine 46 may be useful in predicting the progression and outcome of breast cancer.

E-iii- ***A 9.3-kb DNA genomic sequence, flanking exon 2 to exon 7 of Appl, was isolated and characterized, and was used to generate a targeting construct containing LoxP sites and neomycin resistance sequence.*** In order to understand the significance of the Appl during cellular processes and the potential interaction with other signaling molecules such as AKT2, it is important to analyze this gene in the context of a living animal. In particular, the generation of Appl mice with targeted disruption of Appl in the mammary gland will elucidate the role of Appl in mammary development. Mating Appl mice with other mouse models of breast cancer will reveal the potential of Appl in contributing to breast tumorigenesis.

E-iv-Loss of genetic material (LOH) in the chromosome 17 p13.2 at the microsatellite marker D17S796 has been identified in hepatocellular carcinoma, atypical ductal hyperplasia and in situ ductal carcinoma of breast. Our results shown LOH at the same region in MCF-10F cells treated with the chemical carcinogen benz(a)pyrene (BP). Moreover, microcell-mediated transfer of an intact copy of human chromosome 17 inhibits the transformation phenotype of BP1E cells and PCR-SSCP analyzes show a restoration of the lost material in BP1E-17 neo. These experiments suggest the presence of a TSG in 17p13.2 near the marker D17S796. For this purpose a 940 bp region has been amplified and cloned. The BP1E cell has lost 10-12 bases consisting in a TG repetition. RT-PCR experiments have shown that this region is expressed in MCF-10F, BP1E and BP1E-17 neo. Also we have found that the expressed-sequence tag EST 3179739 matches with the 3'end of this region. Computational analyses show that the

cloned fragment could be the last exon of a bigger peptide. The presence of a 3' splicing site in the putative introns and the ATTAAA region at the 3'end support this idea. The predicted amino acid sequence does not share significant homology with any known protein supporting the idea that this could be a novel protein. Further experiments will be done in order to clone the full-length cDNA and to study the regulation of the expression of this novel gene.

REFERENCES

1. Bouton, A. H., R. B. Riggins, and P. J. Bruce-Staskal. 2001. Functions of the adapter protein Cas: signal convergence and the determination of cellular responses. Oncogene 20:6448-58.
2. Law, S. F., Y.-Z. Zhang, A. Klein-Szanto, and E. A. Golemis. 1998. Cell-cycle regulated processing of HEF1 to multiple protein forms differentially targeted to multiple compartments. Mol. Cell. Biol. 18:3540-3551.
3. Fashena, S. J., M. B. Einarson, G. M. O'Neill, C. P. Patriotis, and E. A. Golemis. 2002. Dissection of HEF1-dependent functions in motility and transcriptional regulation. J. Cell. Sci. 115:99-111.
4. Serebriiskii, I. G., O. Mitina, E. Pugacheva, E. Benevolenskaya, E. Kotova, G. G. Toby, V. Khazak, W. G. Kaelin, J. Chernoff, and E. A. Golemis. 2002. Detection of peptides, proteins, and drugs that selectively interact with protein targets. Genome Res. 12:1785-91.
5. Mailand, N., C. Lukas, B. K. Kaiser, P. K. Jackson, J. Bartek, and J. Lukas. 2002. Deregulated human Cdc14A phosphatase disrupts centrosome separation and chromosome segregation. Nat Cell Biol 4:317-22.
6. Alexandropoulos, K., and D. Baltimore. 1996. Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel, p130Cas-related protein, Sin. Genes Dev. 10:1341-1355.
7. Law, S. F., J. Estojak, B. Wang, T. Mysliwiec, G. D. Kruh, and E. A. Golemis. 1996. Human Enhancer of Filamentation 1 (HEF1), a novel p130Cas-like docking protein, associates with FAK, and induces pseudohyphal growth in yeast. Mol. Cell. Biol. 16:3327-3337.
8. Parkin DM, Bray F, Ferlay J, Pisani P. 2001. Estimating the world cancer burden: Globocan 2000. Int J Cancer. 94: 153-6.
9. Malkin D, Li FP, Strong LC, Fraumeni JF Jr, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, et al. 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science. 250: 1233-8.
10. Jerry DJ, Kittrell FS, Kuperwasser C, Laucirica R, Dickinson ES, Bonilla PJ, Butel JS and Medina D. A mammary-specific model demonstrates the role of the p53 tumor suppressor in tumor development. Oncogene 19:1052-1058, 2000.
11. Ho GH, Calvano JE, Bisogna M, Borgen PI, Rosen PP, Tan LK, Van Zee KJ. 2000. In microdissected ductal carcinoma in situ, HER-2/neu amplification, but not p53 mutation, is associated with high nuclear grade and comedo histology. Cancer. 89: 2153-60
12. Done SJ, Arneson CR, Ozcelik H, Redston M, Andrulis IL. 2001. P53 protein accumulation in non-invasive lesions surrounding p53 mutation positive invasive breast cancers. Breast Cancer Res Treat. 65: 111-8.
13. Done SJ, Eskandarian S, Bull S, Redston M, Andrulis IL. 2001. p53 missense mutations in microdissected high-grade ductal carcinoma in situ of the breast. J Natl Cancer Inst. 93: 700-4.
14. Norberg T, Klaar S, Karf G, Nordgren H, Holmberg L, Bergh J. 2001. Increased p53 mutation frequency during tumor progression--results from a breast cancer cohort. Cancer Res. 61: 8317-21
15. Borresen-Dale AL. 2003. Genetic profiling of breast cancer: from molecular portraits to clinical utility. Int J Biol Markers. 18: 54-6
16. Berns EM, Foekens JA, Vossen R, Look MP, Devilee P, Henzen-Logmans SC, van Staveren IL, van Putten WL, Inganas M, Meijer-van Gelder ME, Cornelisse C, Claassen CJ, Portengen H, Bakker B, Klijn JG. 2000. Complete sequencing of TP53 predicts poor response to systemic therapy of advanced breast cancer. Cancer Res. 60: 2155-62
17. Liu G, Schwartz JA, Brooks SC. 2000. Estrogen receptor protects p53 from deactivation by human double minute-2. Cancer Res. 60: 1810
18. Dumont P, Leu JI, Della Pietra AC 3rd, George DL, Murphy M. 2003. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet. 33: 357-65
19. Liang SH, Clarke MF. 2001. Regulation of p53 localization. Eur J Biochem.268: 2779-83

20. Lambert PF, Kashanchi F, Radonovich MF, Shiekhattar R, Brady JN. 1998. Phosphorylation of p53 serine 15 increases interaction with CBP. J Biol Chem. 273: 33048-53
21. Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E, Fornace AJ Jr. 1999. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J. 18: 6845-54.
22. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell. 87: 953-9
23. Gu W, Roeder RG. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell. 90: 595-606
23a-Jerry, D.J., Kittrell, F.S., Kuperwaser, C., Laucirica, R., Dickinson, E.S., Bonilla, P.J., Butel, J.S. and Medina, D. A mammary specific model demonstrates the role of p53 tumor suppressor in tumor development Oncogene 19: 1052-1058, 2000.
24. Medina D, Kittrell FS, Shepard A, Stephens LC, Jiang C, Lu J, Allred DC, McCarthy M, and Ullrich RL. Biological and genetic properties of the p53 null preneoplastic mammary epithelium. FASEB J. 16:881-884, 2002.
25. Mitsuuchi, Y., Johnson, S. W., Sonoda, G., Tanno, S., Golemis, E. A., and Testa, J. R. Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. Oncogene 18: 4891-4898, 1999
26. Strange, R., Metcalfe, T., Thackray, L., and Dang, M. Apoptosis in normal and neoplastic mammary gland development. Microsc. Res. Tech. 52: 171-181, 2001
27. Meyers, E.N., Lewandoski, M., and Martin, G.R. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination.Nat Genet. 18:136-141, 1998.
28. M.P. Cunninghmn. Giving life to numbers. C A Cancer J. Clin. 47:3-4,1997.
29. Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., LJllrich, A. and McGuire, W.L. Human breast cancer: Correlation of relapse and survival with amplification of the BER-2/neu oncogene. Science 235:177-182, 1987.
30. Escot, C., Theillet, C., Lidereau,, R., Spyratos, F., Champeme, M.-H., Gest, J. and Callahan, R. Genetic alteration of the c-myc proto-oncogene (MYC) in human primary breast carcinomas. Proc. Natl. Acad. Sci. USA 83-4834-4838, 1986.
31. 31-All, l.U., Merlo, G., Callahan, R. and Lidereau, R. The amplification unit on chromosome II q 1 3 in aggressive primary human breast tumors entails the bel-1, int-2 and hst loci. Oncogene 4:89-92, 1989.
32. 32-Theillet, C., Adnane, J., Szepotowski, P., Simon, M, M.P., Jeanateur, P., Bimbaum, D. and Gaudray P. BCL-1 participates in the I lql3 amplification found in breast cancer. Oncogene 5:147-149, 1990.
33. Benjamin CW. Hiebsch RR. Jones DA. Caspase activation in MCF7 cells responding to etoposide treatment. Molecular Pharmacology. 53(3):446-50, 1998.
34. Mor G. Kohen F. Garcia-Velasco J. Nilsen J. Brown W. Song J. Naftolin F. Regulation of fas ligand expression in breast cancer cells by estrogen: functional differences between estradiol and tamoxifen. Journal of Steroid Biochemistry & Molecular Biology. 73(5):185-94, 2000.
35. Mullauer L. Mosberger I. Grusch M. Rudas M. Chott A. Fas ligand is expressed in normal breast epithelial cells and is frequently up-regulated in breast cancer. Journal of Pathology. 190(1):20-30, 2000.
36. Shinoura N. Muramatsu Y. Yoshida Y. Asai A. Kirino T. Hamada H. Adenovirus-mediated transfer of caspase-3 with Fas ligand induces drastic apoptosis in U-373MG glioma cells. Experimental Cell Research. 256(2):423-33, 2000.
37. Buglioni S. Bracalenti C. Cardarelli MA. Ciabocco L. Giannarelli D. Botti C. Natali PG. Concetti A. Venanzi FM. Prognostic relevance of altered Fas (CD95)-system in human. International Journal of Cancer. 89(2): 127-32, 2000.
38. Ueno T. Toi M. Tominaga T. Circulating soluble Fas concentration in breast cancer patients.

Clinical Cancer Research. 5(11):3529-33, 1999.

39. Rubinchik S. Ding R. Qiu AJ. Zhang F. Dong J. Adenoviral vector which delivers FasL-GFP fusion protein regulated by the tet-inducible expression system. Gene Therapy. 7(10):875-85, 2000.
40. Lareef, M.H., Tahin, Q., Russo, I.H., Mor, G., Song, J., Mihaila, D., Slater, C.M., and Russo, J. Transfer of chromosome 17(p13.1) to chemically transformed human breast epithelial cells induces Fas-mediated apoptosis. Proc. Am. Assoc. Cancer Res. 42:1475a, 2001.
41. Owen-Schaub L. Chan H. Cusack JC. Roth J. Hill LL. Fas and Fas ligand interactions in malignant disease. [Review] [116 refs] International Journal of Oncology. 17(1):5-12, 2000 Jul.
42. Bruder JT. Appiah A. Kirkman WM 3rd. Chen P. Tian J. Reddy D. Brough DE. Lizonova A. Kovesdi I. Improved production of adenovirus vectors expressing apoptotic transgenes. Human Gene Therapy. 11(1):139-49, 2000 Jan1.
43. Fan L. Freeman KW. Khan T. Pham E. Spencer DM. Improved artificial death switches based on caspases and FADD.Human Gene Therapy. 10(14):2273-85, 1999Sep 20.
44. Kondo S. Ishizaka Y. Okada T. Kondo Y. Hitomi M. Tanaka Y. Haqqi T. Barnett GH. Barna BP. FADD gene therapy for malignant gliomas in vitro and in vivo Human Gene Therapy. 9(11):1599-608, 1998 Jul 20.
45. Kodaira H. Kume A. Ogasawara Y. Urabe M. Kitano K. Kakizuka A. Ozawa K. Fas and mutant estrogen receptor chimeric gene: a novel suicide vector for tamoxifen-inducible apoptosis. Japanese Journal of Cancer Research. 89(7):741-7, 1998 Jul.
46. Russo, J., Bamabas, N., Zhang, P.L. and Adesina, K. 1996. Molecular Basis of breast cell transformation. Radiat. Oncol. Invest., 3:424429.
47. Russo, J., Calaf, G., Sohl, N., Tahin, Q., Zhang, P.L., Alvarado, M.E., Estrada, S. and Russo, I.H. Critical steps in breast carcinogenesis. The New York Academy of Sciences 698:1-20, 1993.
48. Russo, J., Russo, I.H. 1987 Biological and molecular basis of mammary carcinogenesis. Lab. Invest. 57: 112-137.
49. Russo, J., Calaf, G., and Russo, I.H. 1993. A critical approach to the malignant transformation of human breast epithelial cells. CRC Critical Reviews in Oncogenesis, 4:403-417.
50. Russo, J., Reina, D., Frederick, J. and Russo, I.H. 1988. Expression of phenotypical changes by human breast epithelial cells treated with carcinogens in vitro. Cancer Res. 48:2837-2857.
51. Russo, J.; Russo, I.H. 1987. Role of differentiation on transformation on human breast epithelia cells. Medina. D., Kidwell, W., Heppner G. and Anderson E. (eds.) Cellular and Molecular Biology of Mammary Cancer. New York: Plenum Publishing Co; pp 399-417.
52. Wu, Y; Bamabas, N.; Russo, I.H., Yang, X. and Russo, J. 1997.Microsatellite Instability and Loss of heterozygosity in chromosomes 9 and 16 in human breast epithelial cells transformed by chemical carcinogens. Carcinogenesis, 18:1069-1074.
53. Huang, Y., Bamabas, N., Yang, X. and Russo, J. 1997 Microsatellite Instability and loss of heterozygosity in chromosome 13 and 17 of chemically transformed human breast epithelial cells. Proc. Am. Assoc. Cancer Res.,38: 997a.
54. Calaf, G., and Russo, J. 1993. -Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis, 14: 483-492.
55. Tay, L.K. and Russo, J. 1981 Formation and removal of 7,12- dimethylbenz(a)- anthracene-nucleic acid adducts m rat mammary epithelial cells with different susceptibility to carcinogenesis. Carcinogenesis, 2:1327-1333.
56. Tay, L.K. and Russo, J. 1983. Metabolism of 7,12- Dimethylbenz(a)anthracene by Rat Mammary Epithelial Cells in Culture. Carcinogenesis 4-733-738.
57. Russo, J., Mills, M.J., Moussalli, M.J. and Russo, I.H. 1989. Influence of human breast development on the growth properties of primary cultures. In Vitro Cell Develop. Biol., 25: 643-649.
58. Russo, J. and Russo, 1. H. 1989. A comprehensive approach to mammary carcinogenesis. Proc.

Int. Assoc. Breast Cancer Res. 1: 5.

59. Hu, Y.F., Russo, I.H., Zalipsky, U., Lynch, H.T and Russo, J. 1997 Environmental chemical carcinogens induce transformation of breast epithelial cells from women with familial history of breast cancer. In Vitro Cell. Dev. Biol. 33: 495-498.
60. Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, N.D., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F. and Brooks, S.C. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Research, 50:6075-6086, 1990.
61. Tait, L., Soule, H., Russo, J. 1990. Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell lineMCF-10. Cancer Res., 50:6087-6099.
62. Foumier, R.E.K., and Ruddle, F.H. Microcell-mediated transfer of mun'ne chromosomes into mouse, Chinese hamster, and human somatic cells. Proc Natl Acad Sci USA 74:319-323, 1977.
63. Stanbridge, E.J. Functional evidence for human tumor suppressor genes- chromosome and molecular genetic studies. Cancer Surveys 12:5-24, 1992.
64. Hunt J.D. Evaluation of phenotypic alteration by microcell- mediated chromosome transfer. Analytical Blochem 2338:107-116, 1996.
65. Huang, Y., Bamabas, N., Yang, X. and Russo, J. Microsatellite Instability and loss of heterozygosity in chromosome 13 and 17 of chemically transformed human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 38:997a, 1997.
66. Yang, X., Huang, Y. and Russo, J. Chromosome 17 suppresses the expression of neoplastic phenotypes in benzo(a)pyrene transfon-ned human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 38:3450a, 1997
67. Koi M, Umar A, Chauhan DP, Chen'an SP, Carethers JM, Kunkel TA, and Boland CR (1994) Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N- methyl- N'- nitroN-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res 54:43084312.
68. Lakhani, SR; Collins, N.; Stratton, M.R.; Sloane, J.P. Atypical ductal hyperplasia of the breast: clonal proliferation with loss of heterozygosity on chromosomes 16q and 17p. J. Clin. Pathol 48:611-615, 1995.
69. Grupta P.K., et al. High frequency in vivo loss of heterozygosity in primarily a consequence of mitotic recombination. Cancer Res. 57, 1188- 1193, 1997.
70. Zenklusen J.C., Conti C.J., and Green E.R. Mutational and functional analyses reveal that ST7 is highly conserved tumor- suppressor gene on human chromosome 7q31. Nature Genet. 27, 392-398, 2001.
71. Jacob, M. and Gallinaro, H. The 5' splice site: Phylogenetic evolution and variable geometry of association with U1RNA. *Nucl. Acid Res.* 17: 2159-2180, 1989.

APPENDICES - None